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EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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A METHOD FOR REPEATEDLY SAMPLING THE BLOOD OF THE PORTAL VEIN IN HEALTHY ANIMALS.

By M. A. BLANKENHORN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York, and the Medical Research Laboratory of Lakeside Hospital, Cleveland.)

(Received for publication, July 1, 1926.)

A method to obtain samples of the blood of the portal vein at will would be of extreme value for the study of the phases of intestinal absorption and of liver problems not to mention others. The method here described was devised during the course of a study of the reabsorption of pigments of biliary origin for which the usual laboratory methods entailing laparotomy had proved inadequate. While this method does not permit sampling entirely at will, it does permit repeated tapping of the vein in the healthy and unanesthetized animal.

The plan pursued is to place a silver tube within the body of the animal. For weeks or months after the animal has recovered from the operation this tube will serve as a guide for a puncture of the portal vein.

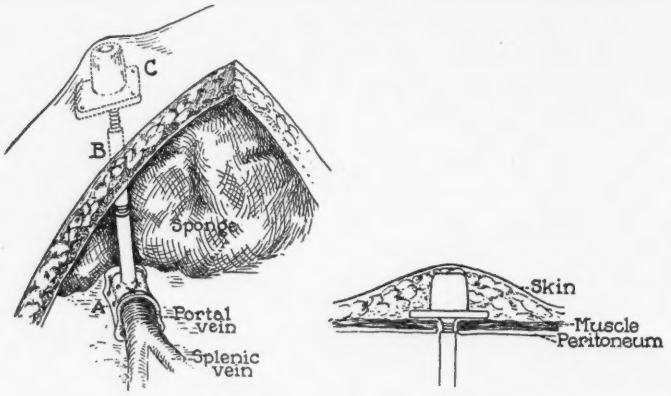
To be successful as a guide to tapping the portal vein the tube must be in contact with it at one end. The other end must be palpable beneath the skin and easily entered by a needle large enough for the withdrawal of blood. The device must be well tolerated by the animal and do no injury to the abdominal viscera.

Such a cannula was made of silver in the form illustrated in Text-fig. 1. For convenience in installing it was made in three parts.

A is a saddle-shaped plate which spans the portal vein and is held there by large silk threads. *C* is a plate with a nipple on it which is fastened into the abdominal wall near the costal margin so that it is covered only by skin. *B* is a hollow stem that connects *A* and *C* by screwing into both these parts when they are in place. This stem is fitted with a right hand screw on one end and a left hand screw at the other so that turning in the one direction engages both the end

pieces (*A* and *C*) and draws them closer together. This screw arrangement not only facilitates assembling the apparatus but regulates the length of the cannula so that it will fit dogs of various sizes.

In practice the details of operation to install the cannula are as follows: Under ether, a midline incision is made from the xyphosternum to one-third the distance to the pubis; self-retaining retractors are put in and wide retraction made. A sand bag under the lumbar spine increases the exposure and large moist sponges are packed into the abdomen to keep the intestines out of the field. The portal vein in the dog is retroperitoneal in about one-half its circumference so a small amount of dissection is required to permit the saddle *A* to span the vein without tension. When the dissection is done two very thick silk threads are passed entirely under the vein at the cephalic and caudal ends of the dissection. These threads are quite long and both ends of each are brought up and out of the inci-



TEXT-FIG. 1.

sion. Each thread is now put through a hole in the four corners of the plate and the plate, held by a hemostat, is lowered along the threads and into place. When it is in place the opposing threads are tied over the top of the saddle. These ligatures keep the saddle in place without compressing the vein at any point.

The plate carrying the nipple *C* must next go in. To guard against the infection that commonly develops around foreign bodies the skin must not be injured and the button must be out of the line of the main incision. To make a bed for the button, the right belly wall is everted as far as possible, with the retractor out. The end of the ninth costal cartilage is clipped off and an incision made from within outwards through the muscles in the line of the ribs, care being taken not to penetrate the skin. With the finger in this incision a pocket is made by blunt dissection of the subcutaneous fascia that will be large enough to contain the plate. When the bed is prepared the piece of silver is pushed through the muscle

incision and held by an assistant, while the operator closes the button-hole-like incision of the muscles with heavy mattress sutures of silk. It is imperative to have this repair so well done that there is no strain on any thread and that the plate retains its subcutaneous position.

It was found necessary to employ a rather large plate to guarantee a firm and permanent fixation to the parietal wall. The nipple upon the plate must also be of such size that the skin is lifted slightly by it, since otherwise the local swelling incident to the healing process may make it difficult to find. Two of the experiments recorded as failures were unsuccessful because the nipple could not be found with a needle even though the plate was firmly fixed.

With the two ends of the apparatus now in place an assistant grasps each with a hemostat to keep them in proper alignment while the operator screws in the connecting stem with his fingers, to what appears to be the proper length for an avoidance of tension when the animal is once more upon its feet. The abdomen is then closed in three layers with silk. The entire operation must be done with strict surgical care against infection for the retained foreign body predisposes to localized peritonitis or abscess.

When the animal is well recovered and the incision healed, which is usually within a week, the tappings can be begun. If the length of the stem of the silver tube is correct for the animal, and there is no strain on the vein or on the abdominal wall, the device is well tolerated, and the animal appears normal. If free from infection the skin fits flatly over the button. Such serum as may exude about the plate has free access to the abdomen and drains away.

The technique of tapping is as follows: The animal is placed on the left side, and the skin over the button is shaved and swabbed with iodine. A No. 17 lumbar puncture needle with stylette is thrust through the skin and down the cannula until close to the vein, when the stylette is withdrawn and a syringe attached on the needle. With suction made the needle is now advanced till it enters the vein, when blood flows into the syringe. The distance from skin to vein must already have been marked on the needle. It will be known from the length of the cannula as installed. A convenient aspirating apparatus is a 20 cc. Record syringe fitted with a rubber stopper and tube so that the operator can make suction with his mouth while manipulating the needle. It is well to have a little oxalate solution in the syringe and the needle, for clotting occurs very readily, partly on account of the length of the needle and partly because blood or serum may enter the needle from the cannula before the vein is entered. Precautions to guard against infecting the vein at the time of the tapping will prolong the usefulness of the device.

To employ a needle with stylette complicates the process of puncturing the vein and doubtless consumes time at a critical moment when clotting is most likely to occur, but on the other hand the stylette keeps the needle relatively free from the clotted lymph or fibrin that fills the lumen of the cannula. A further detail in construction of the cannula not shown in the illustration is the presence of minute holes in the walls of the cannula stem. These holes permit the escape of fluid or fibrin when a needle is thrust into the cannula.

Thus far the apparatus has been installed in thirteen dogs. Of seven instances in a series at Lakeside Hospital, Cleveland, three were successful, and from one to seven specimens of blood were obtained from each dog. One animal was tapped 7½ months after operation. Of six instances studied at The Rockefeller Institute four were successful and two unsuccessful. The form of the apparatus pictured was developed during the course of work at the Institute.

The chief limitations of the method are referable to thrombosis of the vein. This is a result of the contact of metal with the vein, the continuous movement of the cannula upon it with the breathing, and the repeated trauma from the aspirations. The thrombus which gradually develops does not entirely obstruct the vein but tends to block the end of the needle while permitting blood to flow round about it.

It is probable that celluloid will prove better than silver as a material from which to make the cannula. This material is apparently better tolerated than silver and had a certain amount of flexibility, yielding to respiratory movements with less trauma to the vein. The most successful series of tappings were accomplished with a celluloid cannula, at the Lakeside Hospital. This cannula eventually came apart by some solvent working on its cemented joint.

It is highly probable that methods to sample blood from deep lying veins have been described but the writer is aware of but one such in the literature. London[1] described a method that involved intubation of the splenic vein with a glass tube which was continued through the abdominal wall with rubber and kept closed with a dressing. The success of his arrangement was not reported upon.

The writer is indebted to Dr. P. D. McMaster of The Rockefeller Institute and to Dr. Graham and Dr. Beck of the Department of Surgery of Lakeside Hospital for assistance in these experiments.

SUMMARY.

A method is described for repeatedly sampling the blood of the portal vein in healthy animals. The method has been employed in thirteen dogs with success in eight.

[1] London, E., *Quart. J. Exp. Physiol.*, 1923, suppl., 173.

ON THE ABSORPTION OF BILE PIGMENTS FROM THE INTESTINE.

By M. A. BLANKENHORN, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York, and the Medical Research Laboratory of Lakeside Hospital, Cleveland.*)

(Received for publication, October 4, 1926.)

Clinical and experimental studies of the distribution and elimination of bile pigments in man have furnished valuable information as to the state of the liver and the function of bile ducts. Like studies aimed at an understanding of the condition of the blood with special reference to hemoglobin economy have not been so successful although it is well understood that there is an essential relationship between bile pigment and blood pigment (1). That blood destruction finds expression in bile pigment formation (2) and that true bile pigment, as distinct from that of extraneous origin, has no other source than hemoglobin (3) are facts now generally accepted; but as yet no certain quantitative relationship between the two has been established. The chemical process, taking place within the body, which leads to a conversion of one pigment into the other is entirely unknown and other factors less fundamental in nature interfere with our using bile pigment, or any of its derivatives, as a means to measure blood destruction. Among the most important of these factors is the possible reabsorption of bile pigments from the intestine, a reabsorption which might change the bile output or add to the store of hemoglobin. There is much clinical and some direct experimental (4) evidence that such reabsorption does occur. But the final proof, namely a demonstration of the presence of bile pigments in the blood or lymph coming away from the intestine and its removal by the liver, has been wanting. To supply this evidence has been the object of the present investigation.

The blood of the portal vein has been examined frequently for bile pigments, but always under the complex conditions which result from opening the abdomen; and no bile pigments have been found by any observer.

The method I have employed here is new and makes the portal vein accessible to puncture almost at will, weeks after the healing of the operative wound[1] necessary for the introduction of the puncture apparatus. To render the portal vein thus accessible a hollow silver tube is placed within the abdomen, one end being fixed to the vein while the other end lies beneath the skin of the belly wall where it is easily found on palpation prior to introducing a needle. The needle is thrust through the skin, through the tube directly into the vein. The complete details of this method are given in another paper.

In the present study seven dogs were successfully operated upon and furnished nineteen different specimens of portal vein blood to be tested for bile pigments. Six of the animals were also operated for "altercursive intubation" of the common bile duct after the manner of Rous and McMaster (5), so that the pigment content of the intestine could be controlled at will. Both operations were done in each instance at a single sitting.

When the operated animals were well recovered and appeared to be healthy, they were bled from jugular vein and portal vein at the same occasion and the specimens examined for bile pigments. The animals equipped with "altercursive intubation" were, after preliminary bleeding, adjusted for bile collection so that no bile entered the intestine. Stools and urine were collected daily and examined for bile pigment. When stools were entirely acholic, the animal was again bled from portal vein and jugular vein. Following this bleeding, bile was permitted to return to intestine and when stools showed the return of pigment bleeding was done again. In accord with this plan portal vein blood and jugular vein blood were compared as to their bile pigment content under conditions of choice in so far as there was or was not bile within the intestine. In but one dog were we able to get portal blood of all three phases in the same animal.

Blood specimens were tested for bilirubin by the diazo reaction, but quantitative tests were not possible on account of the minute quantities found. Urobilin was tested by the Schlessinger fluorescence method and quantitative determinations made according to a plan

[1] Ether anesthesia was used in every instance where an animal was operated upon, and anesthesia continued throughout the operation.

proposed by Elman and McMaster (6) and changed somewhat to deal with very small amounts as found in blood and lymph.

In brief these animals at no time showed any bilirubin in portal vein blood or jugular blood, nor even any yellow coloring of the plasma. On the other hand urobilin was always present in portal vein blood whenever there was bile in the intestine and always absent from portal vein blood when bile was excluded from intestine. Urobilin was found occasionally in jugular blood but only when also in portal blood and always in less amounts. The amount of urobilin in portal blood averaged .008 mg. per cc., and that of the jugular blood .0006 mg. per cc.

The protocol given herewith is of the animal from which portal blood in all three phases was obtained; that is to say, portal blood when there was bile in the intestine, portal blood when there was no bile in the intestine, and portal blood when bile had been returned again to the intestine.

Protocol.

Dog 4, dachshund, 8 kilos.

October 22, 1925. Ether anesthesia. Operation for portal vein sampling and for "altercursive intubation" of common duct.

1st day after operation. Dog active, wound clean.

4th day after operation. Tapped portal vein for 30 cc. Portal blood was negative for bilirubin, positive for urobilin (.0036 mg. per cc.). Jugular blood negative for bilirubin, positive for urobilin (.00054 mg. per cc.). Clamp put on for bile collection.

27th day after operation. Stool and urine negative for urobilin and bilirubin. Bile urobilin-free. Tapped portal vein, 10 cc., negative for bilirubin and urobilin. Jugular vein blood negative for bilirubin and urobilin. Clamp put on for return of bile to intestine.

29th day after operation. Stools contain much urobilin. Urine small amount. Tapped portal vein, 6 cc., with considerable difficulty. Bilirubin negative, urobilin positive (.0018 mg. per cc.). Jugular blood negative for bilirubin, negative for urobilin.

Lymph Absorption.

Absorption by way of the lymph channels was next investigated and lymph obtained by intubating the thoracic duct in the neck was examined for bilirubin and urobilin under various conditions of

intestinal absorption. In every instance dogs were used, the animals being under ether anesthesia throughout the operation and the period of collection. Animals that had been fasting, and hence presumably not digesting and absorbing, animals that were liberally fed, and animals with enteric injections of bile and chyme containing bile were examined. Lymph specimens were tested for bilirubin and urobilin and compared with specimens of jugular vein blood in the same manner as was portal vein blood in the experiments above.

Eleven dogs that had fasted from 1 to 3 days but had abundance of water were examined. The lymph in five animals contained bilirubin and in six gave no test. Meanwhile the jugular blood in no instance gave a test for bilirubin. This same lymph tested for urobilin was positive in ten instances (average .011 mg. per cc.) while the blood was positive for urobilin in nine specimens (average .003 mg. per cc. or about $\frac{1}{4}$ as much).

Twelve dogs liberally fed and watered 2 to 5 hours previous to the experiment were tested in the same manner. Five of these animals were fed a meal composed of cooked beef lungs, bread, and 200 gm. of lard containing 20 mg. of pure bilirubin dissolved in the lard. Lard has been found to be a good solvent for bilirubin and in the above proportion is intensely yellow so that it can be diluted many times and still give a positive diazo test. Furthermore such lard can be emulsified without losing its bilirubin if kept in a neutral or slightly acid medium. It was thought that bilirubin thus protected from gastric or intestinal digestion might be absorbed into the lacteals after the manner of other fat-soluble dyes. Seven of the twelve dogs showed tests for bilirubin in lymph and five were negative. The positive tests were of the same order of magnitude as in the fasted animals, that is not enough to be measured. At the same time no animal showed bilirubin in jugular blood. The lymph of these twelve animals in every instance contained urobilin (average .0585 mg. per cc.), while the blood was eleven times positive, averaging .00531 mg. per cc. From this it can be seen that feeding had increased very materially the amount of urobilin of lymph and blood, but had practically no effect on bilirubin of either.

Six of the fasting animals tested for absorption by way of lymph were injected with bile containing mixtures directly into the duo-

denum, either by a previously established "altercursive intubation" or by syringe and needle into the exposed gut. Two of these animals received bile-stained chyme removed from another dog supposedly at the height of digestion.

This procedure had little or no effect on the bilirubin content of jugular vein blood for whereas all six animals had no bilirubinemia prior to injection only one showed any bilirubinemia after the injection and that one in amounts too small to be measured. Breast lymph and mesenteric lymph (collected in a manner shown below) also showed little or no change, being practically free of bilirubin at all times.

The injection did, however, cause urobilin to increase, and the jugular vein blood of six animals which, before injection, were three negative and three positive, after injection became five positive and one negative. Breast lymph was similarly affected as shown by Table I.

These experiments to investigate the absorption of pigment by way of the lymph (twenty-three in all) showed very clearly that when intestinal absorption was active urobilin was present in lymph in amounts greater than in systemic blood, and furthermore that when the concentration of pigment in the intestine was increased by injection, urobilin of the lymph was also increased.

It must be noted, however, that bilirubin was present in the lymph in twelve out of twenty-three tests, occurring slightly oftener in fed animals and injected animals than in fasting animals as shown by Table I. This does not indicate absorption of pigment, for such amounts of bilirubin might come directly from the liver by way of the liver lymphatics. The anesthesia did not cause bilirubin to appear in lymph for the general tendency was in all cases towards lesser amounts of pigment as the ether was prolonged. The observation that injections of bilirubin into the intestine did not increase the bilirubin content of the lymph argues very strongly that bilirubin of the lymph was not absorbed bilirubin.

To test the matter of the source of this pigment more critically five animals were operated in a manner to expose the lymphatics of the small intestine in the mesentery just as they enter the receptaculum chyli.

TABLE I.

A. Absorption by Portal Vein. Comparison of Bile Pigment Content of Portal Vein Blood and Jugular Vein Blood in Healthy Animals.

B. Comparison of Bile Pigment Content of Breast Lymph and Jugular Vein Blood of Eleven Fasting Dogs.

C. Comparison of Pigment Content of Breast Lymph, Mesenteric Lymph, and Jugular Vein Blood.

	A		B		C		
	Seven healthy animals		Eleven fasting dogs		Twelve fed dogs		
	Portal vein	Jugular vein	Breast lymph	Jugular vein	Breast lymph	Mesenteric lymph	Jugular vein
Bilirubin positive	0	0	5	0	7	3	0
Bilirubin negative	19	19	6	11	5	2	12
Urobilin positive	9 (Average .008 mg. per cc.)	8 (Average .0006 mg. per cc.)	10 (Average .011 mg. per cc.)	9 (Average .003 mg. per cc.)	12 (Average .0585 mg. per cc.)	5	11 (Average .0053 mg. per cc.)
Urobilin negative	*3	**4	1	1	0	0	1

* In these three instances the animal was acholic on account of bile diversion by way of "altercursive intubation."

** In three instances the animal was acholic on account of bile diversion.

D. Absorption by Way of Lymph. Comparison of Lymph with Jugular Vein Blood before and after Injection of Bile into Intestine.

	Six injected dogs					
	Before injection			After injection		
	Jugular vein	Breast lymph	Mesenteric lymph	Jugular vein	Breast lymph	Mesenteric lymph
Bilirubin positive	0	2	2	1	3	2
Bilirubin negative	6	3	0	5	2	0
Urobilin positive	3	3	2	5	4	2
Urobilin negative	3	3	0	1	1	0

This was done under ether anesthesia which was continued the entire time of the experiment. The intestines were protected from drying by large sheets of rubber dam and all bleeding avoided as carefully as possible. To make a good exposure the abdominal walls were cut down transversely between heavy wire ligatures and all bleeding points tied or clamped. A large lymph channel was then cannulated with a fine glass tube bent at a convenient angle and with a bulb blown in the stem to hold about 3 cc. When the cannula was in place suction applied by a rubber tube caused the cannula and its bulb to fill with lymph. The full bulb was then emptied with a Wright's pipette and in this manner as much as 30 cc. was collected for comparison with blood from the jugular vein.

Of five such specimens three gave positive diazo tests for bilirubin (the greatest being .02 of the van den Bergh unit). While the blood from jugular vein was negative.

All five contained urobilin and averaged .085 mg. per cc. or about the same order of magnitude found in thoracic duct lymph.

This evidence is very suggestive that small amounts of bilirubin were absorbed, for lymph collected in this manner could come from nowhere save the intestine. There remains, however, the possibility that this small amount of bilirubin could have been formed by the peritoneal membranes of the intestine from hemoglobin free in the belly for it is impossible to say that there was no bleeding and the duration of the experiment—*i.e.*, several hours—might possibly be occasion for bilirubin to be formed. The evidence as to urobilin on the other hand is conclusive that this pigment is absorbed from the intestine into the lymphatics.

COMMENT.

The experiments reported here require little comment by way of explanation and even less in interpretation. The observations on the portal vein blood and the jugular vein blood of the seven normal dogs are conclusive—that bilirubin is not and that urobilin is absorbed from the intestines. The proposition that absorbed pigment is in part or altogether removed by the liver from the blood is in fact a corollary to the proof that urobilin is absorbed at all. Such a function of the liver is further suggested by the fact that portal vein blood—as well as lymph from the intestine—always contains more urobilin than does blood obtained elsewhere—that is to say distal to the liver as located in reference to intestine. It is not vital to this paper to know what

becomes of biliary pigments once they are absorbed from the intestine. It is interesting to note that blood that has been through the liver contains much less of this pigment than do blood and lymph coming direct from the intestine. In none of these experiments was anything observed at variance to the idea that urobilin absorbed from the intestine appears in urine and in bile.

Table I is appended to show in condensed form all the data mentioned in the text as well as many other details omitted above for the sake of brevity. The table is self-explanatory.

The writer is indebted to Dr. P. D. McMaster of The Rockefeller Institute for assistance in planning and executing these experiments.

CONCLUSIONS.

Bilirubin as such is not reabsorbed from the intestine by way of the portal vein in healthy animals. Bilirubin may be absorbed from the intestine by the lymphatics but only in minute amounts.

Urobilin is reabsorbed from the intestine by way of the portal vein and by way of the lymphatics.

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SOME OBSERVATIONS ON THE LYMPH AND BLOOD VESSELS OF THE RABBIT TRACHEA.

With Particular Reference to Their Rôle in the Pathogenesis of Experimental Lobar Pneumonia.

By BEATRICE CARRIER SEEGAL,* M.D., and DAVID SEEGAL.**

(*From the Department of Pathology, Harvard University Medical School, Boston.*)

Plates 5 and 6.

(Received for publication, September 16, 1926.)

Blake and Cecil (1) in 1920 presented evidence to show that in lobar pneumonia the pathological process in the lung parenchyma is secondary to a lymphangitis in the submucosa of the main bronchi near the hilum. This point of view is supported by Winternitz, Smith, and Robinson (2) who, by the injection of India ink into the recently sacrificed rabbit's tracheal submucosa, believed that they had demonstrated an unusually rich lymphatic network which was in communication with the lymphatics at the tracheobronchial junction, these in turn communicating with lymphatics leading to the parenchyma of the lung.

The injection method of Winternitz and his colleagues consisted of the insertion of a fine hypodermic needle beneath the mucosa of the trachea. With very gentle pressure on the plunger a rich plexus of vessels was uniformly outlined by the injected India ink. The photomicrographs which accompany this paper demonstrate a profuse anastomotic network of ink-filled channels in the submucosa of the trachea.

In the course of experiments to determine the routes of absorption of various dyes from the pericardial cavity we attempted to outline the peritracheal lymphatics and to repeat the injections reported by Winternitz, Smith, and Robinson.

* Fellow of the National Research Council.

** James Jackson Cabot Fellow.

Experimental Methods and Data.

Rabbits were killed, with an occiput blow. The trachea was then bluntly freed from the surrounding tissues. An incision was made anteriorly in the midline of sufficient length to allow for the injection procedure. A fine 27 gauge platinum hypodermic needle attached to a 2 cc. syringe containing either full or half strength Higgins' India ink was carefully inserted beneath the tracheal epithelium. With gentle pressure on the plunger a rich plexus of ink-filled vessels immediately sprang into view. It was important to avoid holding the trachea on the stretch for this impeded the filling of the channels. With proper pressure and a few changes in the site of injection the entire submucosa of the trachea was outlined by the short, stubby, richly anastomosing ink-filled channels (Fig. 1).

In the midst of one of these injection experiments it was noted that some of the dye was filling a vessel about 1 mm. in diameter lying in the supporting tissue, parallel to and to the left of the trachea. Another vessel filled with ink, smaller than the first, lay in the loose tissue to the right of the trachea. With the aid of the dissecting microscope the smaller of the two channels was traced into the peritracheal tissue, whereas the larger was found to run along the entire length of the trachea, receiving branches from that organ and emptying into the superior vena cava just before this vessel entered the right auricle. Particles of ink were found in the superior vena cava and the right auricle.

Another preparation was made in the following fashion. The rabbit was killed and the trachea slightly freed from its surrounding structures. The vein parallel and to the left of the trachea, referred to above, was identified, tied towards the heart, and injected towards its origin. In a few moments the rich plexus of vessels in the tracheal submucosa became partially filled with the India ink and many veins from the angle of the jaw to the clavicle became stained with the dye.

In a third experiment the rabbit was sacrificed by an occiput blow, the entire trachea removed and slit in the anterior midline. The preparation was then inspected under the dissecting microscope. A plexus of short, stubby, richly anastomotic blood vessels ramified in the submucosa. These vessels were identical with the network which had been injected with ink in the previous experiment and were similar to the channels described by Winternitz and his coworkers as lymphatics.

The hypodermic needle was then inserted into the submucosa and an attempt was made to demonstrate the presence of lymphatics as distinct from the blood vessels. With the aid of a dissecting microscope it could easily be seen that the ink granules entered the meshwork of vessels and pushed the blood cells before them. Repeated attempts to inject lymphatics always met with the same results although five rabbits were used and each trachea subjected to many injections under the microscope.

Pieces of normal rabbit trachea and of tracheæ injected with India ink were fixed in Zenker's and stained with Giemsa.

Examination of the normal trachea demonstrated that the vascular arrangement is of an unusual order (Fig. 2). An extremely rich supply of very thin walled anastomosing blood vessels ramifies in the submucosa. In some sections a single vessel extends about one-fourth the circumference of the trachea. Many little outpocketings designating the junction of tributaries are observed in these larger channels. The walls of these vessels consist of a single layer of endothelial cells with a very delicate connective tissue stroma which is best brought out with Mallory's aniline blue stain. If these thin walled vessels are not filled with blood it is very difficult to distinguish them from lymphatics. It is possible, however, to identify lymphatics and the submucosa but they are of very small calibre, relatively few in number when compared with the blood vessels, and the supporting connective tissue for the latter is slightly more prominent than that for the lymphatics.

The large bronchi contain the same arrangement of blood vessels.

Sections through the trachea which have been injected with India ink present one of two pictures. In the one the red and white cells of the vessels have been completely replaced by ink particles and photomicrographs of these regions resemble those described by Winternitz, and his collaborators, as being ink-filled lymph channels. In the other group of sections, ink granules and blood cells may be seen mingled in the same vessel (Fig. 3).

The human trachea[1] was examined to ascertain whether there existed in the submucosa a vascular supply comparable to that

[1] We wish to thank Dr. Charles Connor of the Pathology Department of the Peter Bent Brigham Hospital for his aid in obtaining this material.

found in the rabbit. The thickness of the mucosa made it impossible to study the vascular channels under the dissecting microscope. Nevertheless, strips of the normal human trachea were subjected to the technique of a blind stab and injection into the submucosa. The results derived from this procedure were unexpectedly satisfactory. Grossly it could be readily seen that two independent types of vessel plexuses existed. Sometimes the one type and sometimes the other was injected. One plexus was finer than the other. Microscopic examination of sections prepared from the injected areas showed these channels to be lymphatics which are smaller and less numerous, than the blood vessels (Fig. 4).

The second type of plexus injected is made up of small blood vessels. These vascular structures bear no resemblance to the rich blood supply found in the rabbit trachea.

SUMMARY AND CONCLUSIONS.

1. Winternitz, Smith, and Robinson have offered as experimental evidence in support of the lymphatic path of infection in lobar pneumonia, a series of injection experiments of the rabbit's tracheal submucosa.

What these investigators supposed to be lymphatics are shown by us to be an unusual type of venous supply consisting of short, wide, and richly anastomosing vascular channels.

2. There is little homology between the structure and distribution of the lymph and blood vessels in the human and rabbit trachea.

3. Injections of pneumococci by the tracheal route to produce lobar pneumonia in the rabbit include an intravenous administration of these organisms when the mucosa is pierced or injured.

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EXPLANATION OF PLATES.[2]

PLATE 5.

FIG. 1. Blood vessels of the submucosa of the rabbit's trachea injected with India ink. Preparation cleared by the method of Spalteholz.

FIG. 2. Blood vessels in the submucosa of the rabbit trachea.

PLATE 6.

FIG. 3. Blood vessels in the submucosa of the rabbit trachea partially filled with India ink.

FIG. 4. Submucosa of the human trachea. The lymphatics are filled with India ink.

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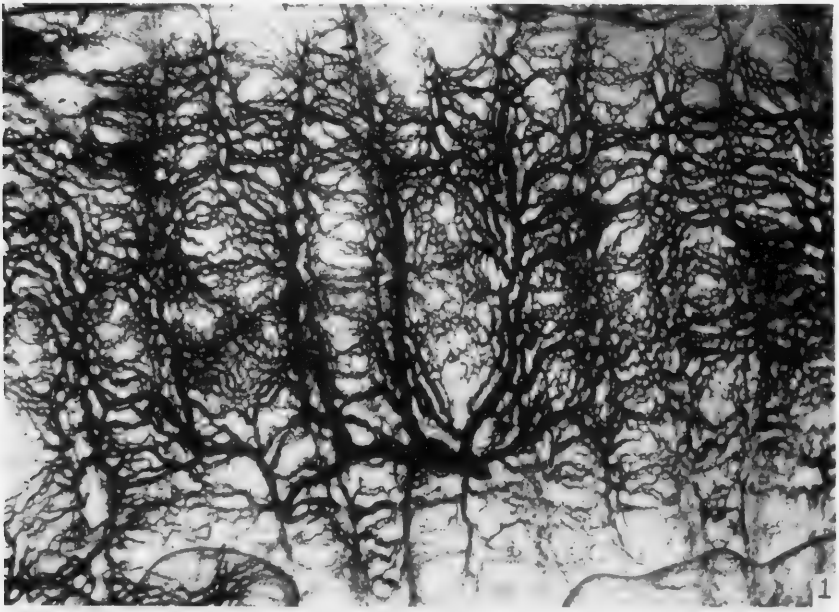
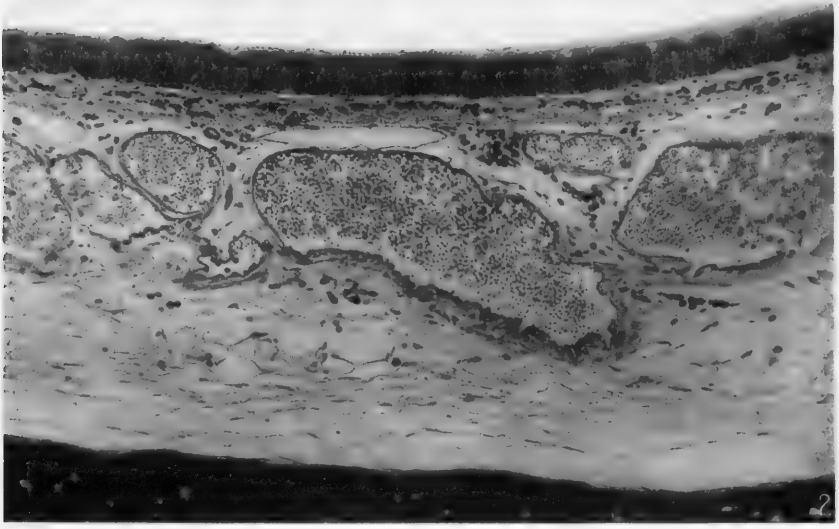
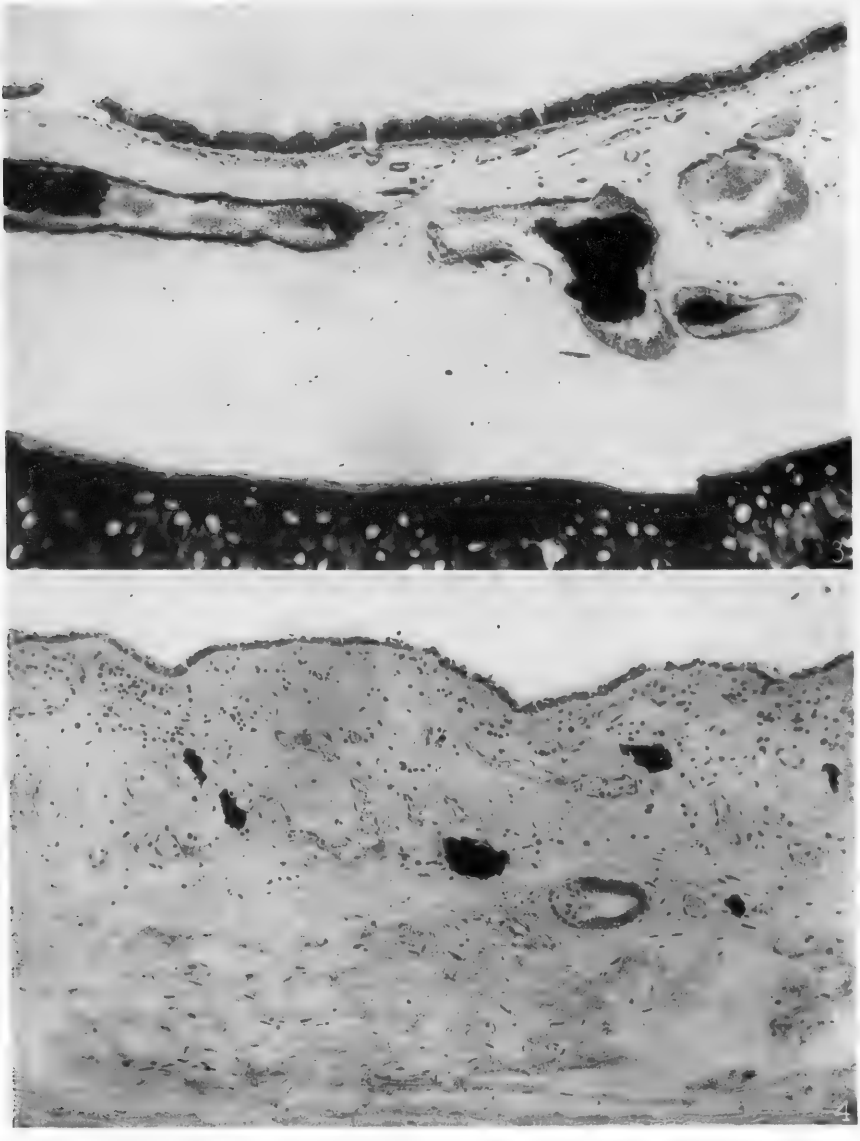
[2] We are indebted to Dr. S. B. Wolbach for the photomicrographs.





(Seegal and Seegal: Lymph and blood vessels of rabbit trachea.)



(Seegal and Seegal: Lymph and blood vessels of rabbit trachea.)

EXPERIMENTAL EPIDEMIOLOGY OF TUBERCULOSIS.

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The pioneer studies in experimental epidemiology made with mouse typhoid at The Rockefeller Institute by Flexner (1), Amoss (2), Lynch (3) and the extensive experiments of Webster (4) and Pritchett (5), which together with the work of Topley (6) in England initiated the new field of experimental epidemiology, have suggested studies along similar lines in the experimental epidemiology of tuberculosis. At the suggestion of Professor Eugene L. Opie, I have undertaken some preliminary experiments in artificially induced epidemics of tuberculosis in guinea pigs.

Studies with tuberculosis present numerous difficulties not encountered in a more rapidly promulgated disease such as mouse typhoid. The duration of the disease, the nature of the infection, the difficulty of spontaneous transmission from animal to animal and the chronicity of the resulting infection make experimental epidemiological studies in tuberculosis a matter of years. Greater hazards than usually occur in animal experimentation are involved in maintaining large groups of guinea pigs alive for months in the same cage.

In the course of the preceding year it was noted in this laboratory that several instances of spontaneous tuberculosis occurred in guinea pigs and rabbits which either had been injected with some innocuous material in the course of other experiments or had been caged merely in the same room with tuberculous animals. These are reported in detail later in this paper. It seemed essential to determine under experimentally controlled conditions the frequency of the occurrence of spontaneous tuberculosis and the factors in its spread. The results of these preliminary studies are presented in this paper.

Reports of spontaneous tuberculosis in mammals other than man, cattle and swine are numerous. Calmette (7) in a review of the literature of spontaneous

tuberculosis in mammals states that it is very rare in the horse, ass, goat, sheep, cat, rabbit, guinea pig and rat but may occur in these animals if they are exposed for long periods to tuberculous cattle or man. It is well known that tuberculosis is very frequent in captive monkeys. Both human and bovine strains have been isolated. Animals in the wild state are said never to contract tuberculosis. While examining ground squirrels captured in the wild state, for the plague, five instances of natural tuberculous infection of the bovine type were observed by McCoy and Chapin (8).

In the Philadelphia Zoological Garden, Fox (9) found tuberculosis in nearly all the vertebrates examined. In a series of 1860 autopsies on Mammalia, 14.7 per cent died from naturally acquired tuberculosis. Of all primates autopsied, 48.5 per cent were tuberculous; of Lemures, 26.7 per cent; of Carnivora, 3.5 per cent; of the Rodentia, 2.5 per cent; of the Ungulata, 9.6 per cent; of the Proboscidea, 66 per cent (2 of 3 instances). A study of the pathological types observed reveals the frequency of the "nodular and caseous forms." No fibroid tuberculosis approaching the quiescent type as seen in man and rarely in domestic animals was encountered. Calcareous deposits were extremely rare. The most susceptible tissue of wild animals in confinement is the lung. The probable origin of the infection in all orders of the Mammalia of this series was bronchiogenic in about half of the instances and intestinal and lymphatic in the other half.[1]

Rabbits and guinea pigs exposed in the same room with tuberculous animals for a longer period than 4 months, Koch (10) found not infrequently developed spontaneous tuberculosis. This significant observation made 40 years ago has not been adequately appreciated by workers in tuberculosis. The occurrence of natural infection was in direct proportion to the number of tuberculous animals present in the room. According to Koch, the type of disease in spontaneous tuberculosis in these animals is characteristic. The lungs contain large caseating areas with occasional cavitation and the hilum nodes are enormously enlarged and caseous. This striking picture in sharp contrast with the pathology of induced infections is evidence of the respiratory route of infection. Koch emphatically states that if rabbits or guinea pigs have remained 3 or more months in the same room with tuberculous animals, the experiments in which these animals are used cannot be relied upon, unless spontaneous tuberculosis can be excluded. Isolated instances of spontaneous tuberculosis in rabbits were reported by Strauss (11) and Guérin (12) and an epizootic of spontaneous tuberculosis of the bovine type on a rabbit farm was studied by Rothe (13). He observed that tuberculosis was readily contracted by normal animals when directly exposed to infected cage mates. If, however, a normal animal merely occupied the cage where an infected animal had lived, it did not contract tuberculosis. He fails to describe the pathological anatomy of the disease. Within 4 years, Couland (14) observed seventeen instances of spontaneous tuberculosis in rabbits. The lesions were primarily in the lungs.

[1] A complete review by Eber of the recent literature of tuberculosis in the lower animals may be found in *Ergebn. allg. Path. u. path. Anat.*, 1917, xviii, pt. 2, 1.

One group showed large masses with caseation; another showed discrete tuberculous lesions in the lungs, kidneys and liver. Three of the four offspring of a rabbit that had been inoculated intravenously 8 days prior to parturition developed tuberculosis of the human infantile type (15). On the basis of feeding experiments, in an attempt to determine the pathogenesis of the naturally acquired infection in these animals, Couland concluded that intestinal and mesenteric lesions developed in rabbits that had been fed massive doses of bacilli. With smaller doses, pulmonary lesions similar to those found in spontaneously infected rabbits were observed. The development of these lesions took several months. It is probable that the tubercle bacilli after ingestion penetrated past the epiglottis into the respiratory tract.

In studying the route of spontaneously acquired tuberculosis in a small series of guinea pigs exposed to phthisical patients for several days and then isolated for 30 days, Bartel and Spieler (16) examined sections of the base of the tongue, pharyngeal wall, bronchial, cervical and mesenteric lymph nodes, the trachea and the lungs. The pharyngeal wall showed a microscopic tuberculous lesion in one instance, the lungs and tracheobronchial lymph nodes, occasionally, but the mesenteric lymph nodes most frequently. In exposing guinea pigs to a spray of emulsions of tubercle bacilli during 5 to 10 minutes and again examining the various tissues, Bartel and Neumann (17) found the tracheobronchial lymph nodes most frequently involved although mesenteric and cervical lymph nodes were occasionally affected. Lesions in the tonsils were not present in either the sprayed or exposed animals. The experiments, however, are inconclusive.

Recently, Remlinger (18) confined healthy guinea pigs in the same cage with others having open tuberculous ulcers; 14 per cent of his normal contact animals contracted tuberculosis. Of the normal guinea pigs exposed to infected cage mates that were free from open lesions, 9 per cent developed tuberculosis. Normal guinea pigs confined in cages in which others had recently died of generalized tuberculosis remained well. Of 52 guinea pigs fed for 1 year exclusively on barley soiled with the fecal material of tuberculous guinea pigs, not one developed tuberculosis. Remlinger does not specify the duration of infection in the tuberculous animals at the time feces were obtained nor the quantity of bacilli used in infecting these animals. Sewall and Lurie (19) observed an epizootic of spontaneous tuberculosis in guinea pigs that had been exposed to polluted air and confined for 7 to 18 months in the laboratory of a sanitorium for tuberculous patients. The exact pathology of the lesions is not described.

Spontaneous Tuberculosis in Rabbits and Guinea Pigs.

The occurrence of spontaneous tuberculosis in a rabbit or guinea pig in a laboratory where tubercle bacilli are constantly used for experimental purposes may be due either to naturally acquired infection or, where non-tuberculous material has been injected into

the animal, to wound contamination. Several instances of tuberculosis due to wound contamination have developed in our laboratory in the past year. The discovery of tubercle bacilli in the lesion at the site of inoculation indicates that the infection has entered through the wound. The following instances of spontaneous tuberculosis and tuberculosis due to wound contamination occurred in the general animal room of the Henry Phipps Institute. In these instances a diagnosis of tuberculosis was made on the basis of the pathological findings at autopsy and the presence of acid-fast bacilli in smears made from the involved organs. Animal inoculations were not made.

Rabbit 1[2] was injected with horse serum subcutaneously in the back and died 120 days later. Autopsy—At the site of inoculation in the subcutaneous tissue of the back is a small abscess filled with caseous material which contains numerous bacilli with acid-fast characters. The lungs are studded with grey spots 1 mm. in diameter and several caseous nodules from 2 to 4 mm. in diameter are present. In the right upper lobe close to the hilum, there is a thick walled cavity 1.5 cm. in diameter filled with cheesy yellow material. This infection is probably due to wound contamination.

Rabbit 2 was repeatedly injected with crystalline egg albumin. It died 155 days following the first injection. Autopsy—Tuberculous nodules 8 mm. in diameter are present in both upper lobes of the lungs. The hilum lymph nodes are not enlarged. The spleen and liver are slightly enlarged. There is no lesion at the site of inoculation. This is possibly an instance of bronchiogenic spontaneously acquired infection.

Rabbit 3 died 185 days following the injection of horse serum. Autopsy revealed one large caseous nodule in the left lung close to the hilum. There was no other sign of tuberculosis. This is another instance of spontaneously contracted tuberculosis introduced through the respiratory tract.

Guinea Pig 1 was injected with 12 cc. of horse serum and died 116 days later. Autopsy—At the site of inoculation is a caseous nodule 4 mm. in diameter. The omentum is thickened and studded with yellow caseous spots and fibrous tissue. The spleen is from fifteen to twenty times the normal size and is reddish grey in spots with numerous small caseous areas throughout. The liver is much enlarged and contains numerous yellow areas, 8 mm. in diameter. The splenic lymph node is 1.5 cm. in diameter and is caseous on section. The lungs are studded with grey-yellow spots 3 mm. in diameter. This is obviously an infection due to the accidental introduction of tubercle bacilli at the site of the serum injection.

Guinea Pig 2 was injected with .5 cc. of horse serum intravenously and died 135 days later. Autopsy—The submaxillary and deep cervical lymph nodes are en-

[2] For the purpose of citation animals are numbered consecutively.

larged, firm, from 3 to 4 mm. in diameter and on section show caseous material. The hilum lymph nodes measure 4 mm. in diameter, are matted together and filled with cheesy material. The lungs are studded with confluent grey spots. The spleen is six times the normal size and contains numerous large caseous spots. The liver is much enlarged and contains small yellow, necrotic and irregular patches. One iliac lymph node is enlarged and caseous. This was an instance of spontaneous tuberculosis probably introduced through the pharynx, spreading by lymphatic channels and finally reaching the respiratory tract with general dissemination.

All the animals reported above had been kept in separate cages in the general animal room and tuberculosis was incidentally discovered during the course of experiments not concerned with tuberculosis.

To test the incidence of spontaneous tuberculosis in the general animal room, four cages each containing four guinea pigs were placed in various parts of the animal room. One cage was placed in the northeast corner among rabbits treated with horse serum in the course of other experiments; one among cages containing guinea pigs injected with suspected tuberculous material; another in the southeast corner among a similar group of animals; and a fourth among tuberculous rabbits. Two instances of spontaneous tuberculosis developed in these animals and both were in the group that had been placed among the cages containing tuberculous rabbits.

Guinea Pig 3 lived 132 days after exposure and was killed to obtain serum. Autopsy—The spleen is slightly enlarged and nodular. The liver shows a few yellow spots. In the center of the left lung there is a thick walled cavity containing cheesy necrotic material. A smear taken from the wall of the cavity shows acid-fast organisms. The tracheobronchial lymph nodes measure 2 cm. in diameter, are matted together and are firm. On section they contain caseous material. There is a complicating adhesive pleurisy and chronic pneumonia of the left lung. The portal of entry of the infection in this instance was the respiratory tract.

Guinea Pig 4 died after 278 days. Autopsy—There is no local lesion. The iliac and lumbar lymph nodes are slightly enlarged. The spleen is five times the normal size, friable and contains several caseous areas. The liver contains a few small patches of yellow necrotic tissue from 2 to 3 mm. in diameter. The lungs are studded throughout with grey-white tubercles, with occasional opaque yellow central spots. The tracheobronchial lymph nodes are large, firm and caseous on section.

The portal of entry in both instances was the respiratory tract. It is apparent that bronchiogenic infection is possible among guinea

pigs exposed to tuberculous animals although isolated in separate cages.

Experimental Studies of Spontaneous Tuberculosis in Guinea Pigs.

The following experiments were undertaken to determine how frequently spontaneous tuberculosis occurs under controlled conditions in guinea pigs exposed to tuberculous guinea pigs. Observation was planned to determine: first, the effect of varying the degree of exposure; second, the effect of crowding on the incidence of spontaneous tuberculosis; third, the pathogenesis and pathology of spontaneous tuberculosis acquired by direct exposure to tuberculous animals with "closed" and "open" lesions.

These tests were carried out in a separate animal room in a separate building away from all possible source of outside contamination and exposure other than that introduced directly into the experiment. The room measured 15 × 8 × 10 feet. There was one window 5 × 3 feet. The temperature was kept nearly constant throughout the experiment. At the beginning of August, however, the constant temperature could not be maintained and since deaths from causes other than tuberculosis were numerous, the experiments were brought to a close, 7½ months after their beginning. About 460 guinea pigs were kept under observation. When there was any doubt about the character of the lesions which were found, sections were made and animal inoculations were used to confirm the diagnosis. The diagnosis of every case of spontaneous tuberculosis was confirmed by smears prepared from the organs involved and stained for acid-fast bacilli. Care was exercised to exclude the possibility that guinea pigs had been exposed to tuberculosis prior to their introduction into the experiments. The normal animals were obtained from The Rockefeller Institute for Medical Research at Princeton. The number of normal and infected animals was kept constant throughout the experiments by replacing the pigs as they died. The animals were fed the routine diet of oats, moistened bread and lettuce leaves. The attendant who cared for these guinea pigs was not permitted to handle animals in the general animal room. Workers from the general animal room were excluded and special gowns were worn by those entering the room.

The infecting organism was originally isolated from the lungs of a patient with pulmonary tuberculosis. It had been grown on glycerol agar in this laboratory during 2 years. Glycerol agar transplants 3 weeks old were used in inoculating the guinea pigs. The strain was highly virulent.

The Relation of Spontaneous Tuberculosis to Intensity of Exposure.

The attempt was made to determine whether spontaneous tuberculosis occurs in guinea pigs exposed to cage mates infected intra-

peritoneally with virulent tubercle bacilli; to determine the relation of incidence to intensity of exposure and to compare this with incidence of spontaneous tuberculosis in guinea pigs exposed to guinea pigs with open tuberculous lesions.

Experiment 1.—60 guinea pigs of the same weight and age were divided into five series of twelve each and placed in five large cages arranged in vertical series. Each cage measured 39 inches long by 13 inches high by 14 inches deep. The top and bottom cages held twelve normal control pigs. In Cage 2, three animals were infected with .001 mg. P-15-B, a virulent culture of human tubercle bacilli; nine were normal cage mates. In Cage 3, six were infected and six were normal. In Cage 4, nine were infected and three were normal. The number of infected and normal animals was kept constant by continual replacement as the deaths occurred. After the first replacement, .00001 mg. injected intraperitoneally was used to infect the guinea pigs. The average life of a guinea pig injected with .001 mg. intraperitoneally was from 6 to 8 weeks, with .00001 mg. from 2 to 3 months. In some cages, as many as five successive replacements of infected animals were necessary. In infecting the animals, intraperitoneally, every precaution was taken to avoid contamination of the subcutaneous tissue of the skin so that in no instance did an open lesion develop. All animals were killed at the end of 7½ months.

Results.

In the control cages, there were no instances of spontaneous infection.

Cage 2.—There was one instance of spontaneous tuberculosis. Guinea Pig 4 was killed after 225 days. Autopsy—A mesenteric lymph node is slightly enlarged and contains a few yellow caseous spots. The spleen is three times the normal size. The liver contains a few yellow spots.

Cage 3.—There was no instance of spontaneous tuberculosis. Only three of the normal animals, however, survived the entire period.

Cage 4.—Of the three normal animals, two were found to have spontaneous tuberculosis at autopsy.

Guinea Pig 5 died after 127 days. Autopsy—The mesenteric lymph node measures .5 cm., is firm and on section presents several very small caseous areas. The spleen is four times the normal size and is nodular with one or two yellow-grey caseous spots.

Guinea Pig 6 was killed after 225 days. Autopsy—A mesenteric lymph node measuring .5 cm. in diameter is very firm and fibrous and on section there are within its substance a few small caseous spots. The spleen is five times the normal size, studded with greyish spots, 2 mm. in diameter. The liver shows a few yellow areas.

The incidence of spontaneous tuberculosis in this cage was 66 per cent of the original three animals, but 100 per cent of those that survived the experimental period acquired tuberculosis.

From this experiment, it is obvious that spontaneous tuberculosis occurs among guinea pigs directly exposed to cage mates infected intraperitoneally with human virulent tubercle bacilli. Of a total of thirteen normal animals which survived the entire period of the experiment and which were directly exposed to animals infected intraperitoneally with human virulent tubercle bacilli, 22 per cent acquired tuberculosis. The portal of entry in all of these instances was the intestinal tract and the mesenteric lymph nodes. The greatest incidence occurred in the cage where the intensity of the exposure was greatest. A greater number of instances of spontaneous tuberculosis would doubtless have occurred had the experiment continued longer, since infection occurred in most instances in this and the following experiments in animals exposed to infection during the entire experimental period.

The source of infection in this experiment was probably through fecal contamination of the food. Careful studies were made in attempting to estimate the quantity of virulent tubercle bacilli eliminated in the excreta of infected guinea pigs. The results of these studies will be reported in a separate paper. It was found that guinea pigs infected intraperitoneally as well as subcutaneously eject viable tubercle bacilli in their feces during the last few weeks of their lives. In some instances animals injected intraperitoneally eliminated tubercle bacilli during the 1st week of the infection. When it is considered that during the period when tubercle bacilli appear in the feces, approximately one-sixtieth the quantity of feces excreted by one infected guinea pig during 24 hours is capable of producing tuberculosis when injected subcutaneously into a guinea pig, a rough estimate of the quantity of infected material to which the normal guinea pigs were exposed may be obtained. Although the cages were cleaned three times a week, there was a constant source of contamination present from the feces.

Experiment 2.—Five small cages, each 14 × 17 × 14 inches were arranged in vertical series and thirty guinea pigs divided into five groups of six were placed in the cages. The top and bottom cages each held six normal guinea pigs. In Cage 2 were four normal guinea pigs with two cage mates infected subcutaneously with .01 mg. of P-15-B, the same virulent human strain used in the previous experiment. In Cage 3, three normal and three infected animals were placed; in Cage 4, two

normal and four infected. With the quantity of bacilli used local abscess formation with ulceration occurred in many cases during a period from the 2nd to about the 5th week. Very frequently no ulceration resulted. As the infected animals died, they were replaced by other infected guinea pigs. In many instances, replacements were made four times in the course of the experiment. At the end of 7½ months the experiment was brought to a close as in the first experiment. The animals were kept in the same isolated animal room as those of Experiment 1.

Results.

No instances of acquired infection occurred in the control cages.

In Cage 2, there were two instances of spontaneous tuberculosis.

Guinea Pig 7 died after 222 days. Autopsy—A mesenteric lymph node is 1 cm. in diameter, dense and caseous on section. There are a few yellow spots on the serosal surface of the ileum but no definite ulcerations in the intestinal tract. The spleen is three times the normal size, red, with scattered greyish spots. The liver is much enlarged. The right lobe contains several large areas from 3 to 8 mm. in width, which are yellow and caseous on section.

Guinea Pig 8 was killed after 226 days. Autopsy—A fibrous mesenteric lymph node measures .5 cm. in diameter. The spleen is nodular and three times the normal size. On its surface are a few greyish caseous spots. The liver shows a few minute yellow spots.

Of four normal animals, two or 50 per cent acquired tuberculosis. All four animals had survived about the same length of time.

Cage 4.—One animal developed spontaneous tuberculosis.

Guinea Pig 9 died after 117 days. Autopsy—The left submental lymph node measures 4 mm. across, is firm and on section shows several caseous areas. The deep cervical lymph nodes are matted together in a firm mass measuring 1.5 cm. in diameter and containing several areas of caseation. A mesenteric lymph node measures 5 mm. and is fibrocaseous on section. The spleen and liver are moderately involved.

In Cages 4 and 5 there were no instances of spontaneous infection.

In this experiment, of the eight normal guinea pigs originally placed in the cages which were directly exposed to animals with open lesions during 7 months, three or 38 per cent developed tuberculosis. One died of an acquired tuberculosis 117 days after the beginning of the experiment.

In contrast with the first experiment, the incidence in the second experiment is higher. According to Römer (20) every guinea pig directly exposed during 1 year to infected cage mates with open ulcerations acquires tuberculosis. In his classic experiments on reinfection, his pigs were infected with 5 mg. of bovine tubercle bacilli injected subcutaneously into both thighs. The infecting dose was enormous and the resultant ulcers consequently offered abundant

opportunity for infection to normal cage mates. In our experiment much smaller infecting doses were used and ulcerations were obtained in considerably less than half of the instances. The tendency of guinea pigs to lick the open ulcerations of their cage mates is, of course, an obvious means of transmission of the infection.

The pathogenesis of spontaneous tuberculosis acquired by guinea pigs exposed directly to cage mates having open ulcerations is clear. It is noteworthy that infection *via* the intestinal tract with involvement of the mesenteric lymph nodes not infrequently is accompanied by massive infection of the submental and cervical lymph nodes. This association of the mesenteric and cervical lymph node tuberculosis is significant and is probably analogous to childhood bovine tuberculosis in which these lymphatic structures are primarily involved. Massive involvement of cervical lymph nodes was observed in instances of spontaneous tuberculosis incidentally reported in Römer's work.

The Effect of Crowding on the Incidence of Spontaneous Tuberculosis in Guinea Pigs.

The attempt was made to determine the relation of incidence to crowding by increasing the number of normal animals exposed and keeping the source of infection constant.

Experiment 3.—Four large cages, 40 × 14 × 17 inches, were arranged in vertical series in the animal room containing the other experimental cages. In Cage 1 were placed twelve guinea pigs, six normal and six infected intraperitoneally with .001 mg. of a 3 week glycerol agar culture of a virulent human strain of tubercle bacilli, P-15-B, the same strain used in the other experiments. In the later period of the experiment, infected animals which died were replaced by others in which .00001 mg. was used as the infecting dose. The average duration of life with .001 mg. was 6 weeks; with .00001 mg., 2 or 3 months; with .000001 mg., 3 or 4 months. In Cage 2, were placed eighteen guinea pigs, six infected with twelve normal cage mates. In Cage 3 were twenty-four guinea pigs, six infected with eighteen normal cage mates. In the lowermost cage were thirty guinea pigs; six were infected and twenty-four were normal. The control cages of Experiment 1 served as controls for this experiment as well. The cage used in the present experiment houses twelve pigs without evident crowding. The duration of the experiment was 7 months.

Results.

Cage 1.—There were no instances of spontaneous tuberculosis; four of the original six animals survived during the entire experimental period.

Cage 2.—There were two instances of spontaneous tuberculosis.

Guinea Pig 10 was killed after 210 days. Autopsy—A mesenteric lymph node 1 cm. in diameter is densely fibrous. The spleen is four times the normal size and contains numerous greyish pink spots slightly elevated above the surface, the spots varying from 1 to 3 mm. in diameter.

Guinea Pig 11 at the end of 94 days of exposure gave birth to one animal. It was removed from the cage during the remainder of the experiment and placed in a separate cage with its offspring. 68 days later, it again gave birth to a litter of three. It died after 192 days. Autopsy—A mesenteric node is much enlarged measuring 1.5 cm., is firm on section and contains two small caseous spots. The spleen is five times the normal size; it has a granular surface and contains numerous caseous spots. The liver is slightly enlarged and shows a few scattered small necrotic areas.

Of the twelve normal guinea pigs originally placed in the cage, seven survived during the entire experimental period. The incidence of spontaneous tuberculosis was 29 per cent. The total number of animals used in this cage was forty-seven.

Cage 3.—Of eighteen normal animals originally included in the experiment, twelve survived during the entire period and nine developed spontaneous tuberculosis. In this cage, however, two animals both tuberculous were found partly eaten after death. These animals died 4 months after the beginning of the experiment.

Guinea Pig 13 was killed after 212 days. Autopsy—The mesenteric nodes are enormously enlarged, matted together in a mass 2 cm. long by 1 cm. in diameter, densely fibrous and contain a few areas of caseation. The spleen is three times the normal size; its surface is granular with minute, fibrous, grey-white spots. The liver shows a few small yellow spots. The submental node on the right and the deep cervical node on both sides measure 8 mm. in diameter. They are firm, fibrous and contain minute caseous areas.

Guinea Pig 14 died after 152 days. Autopsy—The peritoneum is coated with a thick greenish white fibrinopurulent exudate. The spleen and liver are coated with exudate but otherwise normal. Buried among the adhesions in the mesentery is a large firm lymph node 1.5 cm. by 1 cm. which cuts with difficulty and on section shows two areas, 3 and 6 mm. in diameter, of caseous material. There is no other evidence of tuberculosis in the body. There is, however, chronic purulent pleurisy and pneumonia and chronic pericarditis.

Guinea Pig 15 was killed after 212 days. Autopsy—The mesenteric nodes are matted together in a mass 2 cm. in diameter. On section the mass is yellowish in

color and densely fibrous. The spleen is three times the normal size and is nodular. There are one or two yellow spots in the liver.

Guinea Pig 16 was killed after 212 days. The mesenteric nodes are matted together in a mass 2 cm. in diameter. On section the mass is yellowish in color and densely fibrous. The spleen is three times the normal size and is nodular. There are one or two yellow spots in the liver.

Guinea Pig 17 died after 132 days. Autopsy—Almost the entire mesentery is occupied by a large mass 1.5 cm. in diameter, irregular in shape and moderately firm. On section it contains a large area of caseation which is readily removed leaving a thick walled cavity. There is a mass of caseous nodes about 1 cm. in diameter in the region of the upper end of the jejunum and another in the region of the cecum. The spleen is slightly enlarged and is nodular.

Guinea Pig 18 died after 197 days. Autopsy—The mesenteric lymph nodes are matted together in a firm mass measuring 1.5 cm. in diameter, and containing a few caseous spots. In the ileum are four or five deep ulcers each measuring 3 mm. in diameter. The floor of the craters is necrotic. The surrounding tissue is deeply congested. The spleen is nodular and measures four times the normal size. The liver contains numerous yellow spots. The lungs contain scattered grey spots measuring from 1 to 3 mm. in diameter with caseous centers. The hilum nodes are slightly enlarged and contain a few caseous spots. A smear taken from the floor of the ulcer contains acid-fast organisms.

Guinea Pig 19 died after 121 days. Autopsy—The mesenteric nodes are matted together in a firm mass 1.5 cm. in diameter and on section contain several small areas of caseation surrounded by dense fibrous tissue. The spleen is four times the normal size and nodular. The liver contains numerous yellow spots. The iliac, lumbar and hepatic nodes are slightly enlarged and contain minute caseous spots. The ileum contains on its mucosal surface several raised hemorrhagic circular ragged ulcers 4 mm. in diameter which extend to the serosa. On the serosal surface directly over the ulcers are small caseous spots 1 mm. in diameter. The lungs contain scattered grey spots 1 mm. in diameter. Both submental nodes measure 1.5 cm. in diameter, are firm, dense, cut with difficulty and on section contain several caseous areas in their centers. The deep cervical nodes are slightly enlarged.

Guinea Pig 20 died after 139 days. Autopsy—Two mesenteric nodes are much enlarged and contain on section small areas of caseation. The liver contains numerous yellow spots. The spleen is slightly enlarged; its surface is granular. The lungs contain scattered grey spots.

In Cage 3, there were nine instances of spontaneous tuberculosis. The portal of entry in all of these was apparently the intestinal route. Several showed involvement of the cervical lymph nodes. Two had tuberculous ulcerations in the ileum, which is a most unusual finding in experimental tuberculosis. Of the twelve normal animals that

survived the experimental period, seven developed tuberculosis. About 60 per cent of the normal guinea pigs which were originally placed in the cage and which survived the experimental period, developed tuberculosis. One guinea pig developed a fatal tuberculosis within 121 days.

Cage 4.—There were four instances of spontaneous tuberculosis in this cage. Of twenty-four normal animals originally placed in the cage, only eleven survived the experimental period.

Guinea Pig 21 died after 80 days. Autopsy—There is a disseminated tuberculosis involving the omentum, liver, spleen and lungs. The hilum nodes are matted together in a large firm mass measuring 1.7 cm. in diameter and on section contain areas of caseation surrounded by dense fibrous tissue. All other nodes appear to be normal. The portal of entry in this instance was probably the respiratory tract.

Guinea Pig 22 was killed after 212 days. A mesenteric node measuring .5 cm. in diameter is dense and on section entirely fibrous. The spleen is three times the normal size and is nodular with minute caseous spots. In all probability the mesenteric lesion had healed.

Guinea Pig 23 was killed after 212 days. Autopsy—A mesenteric node measuring 6 mm. in diameter is densely fibrous on section. The spleen is four times the normal size, is nodular and contains two or three small yellowish spots. The liver contains a single yellow area 2 mm. in diameter. The left submental node measures 4 mm. in diameter and is firm and fibrous on section.

Guinea Pig 24 was killed after 149 days. Two mesenteric lymph nodes are enlarged, each measuring 4 mm. in diameter. On section, they are fibrous and contain one or two minute areas of caseation. The spleen is three times the normal size; its surface is granular and it contains throughout numerous minute grey-white spots. The liver contains two yellow spots, 2 mm. in diameter.

About 36 per cent of the normal animals surviving the experimental period developed spontaneous tuberculosis. In this cage, in three of the four animals that developed tuberculosis, the digestive tract was the portal of entry, but in one instance the infection was possibly bronchiogenic.

44 per cent of the normal animals originally placed in the cages in this experiment developed spontaneous tuberculosis. The results are indicated in Table I. As may be gathered from the table the greatest incidence did not occur in the most crowded cage. Crowding, however, is doubtless a factor in the spread of spontaneous tuberculosis. If in any group of confined guinea pigs exposed to a given intensity

of infection a definite percentage acquire spontaneous tuberculosis, a greater number of instances will appear in the larger groups of animals. This increase in the number of infected animals in the cage results in an increase in the intensity of exposure for other members of the group.

The experimental period, however, was too short to determine any definite relation between crowding and the incidence of spontaneous infection. Although the total incidence of spontaneous disease in this series is greater than the incidence in the other series where crowding was not a factor, the conditions, notably the intensity of the infection, present in the various cages were different.

TABLE I.

Crowding and the Incidence of Spontaneous Tuberculosis.

Cages	No. of normal guinea pigs	No. of guinea pigs experimentally infected	Per cent of guinea pigs that developed spontaneous tuberculosis
			per cent
Control	12	0	0
Cage 1	6	6	0
Cage 2	12	6	29
Cage 3	18	6	60
Cage 4	24	6	36
Total			44

No definite conclusions, therefore, of the effect of crowding on the incidence of spontaneous tuberculosis can be gathered from this experiment.

DISCUSSION.

In the tests as carried out the animals had abundant opportunity for infection. The number of infected animals was kept constant and in all 204 infected animals were observed. At first, an effort was made to replace the tuberculous animals by others that had been infected during an equal time. As the number of deaths increased this, however, was found to be impossible. A careful study of the elimination of tubercle bacilli in the feces has been made in order to obtain some estimate of the degree of exposure

from this source (20). Some animals infected intraperitoneally discharge tubercle bacilli during the 1st week and there is a considerable elimination of tubercle bacilli during the last few weeks of life when a general dissemination of the tuberculosis occurs. A suspension made from one fecal bolus passed by a guinea pig during the period when it eliminates tubercle bacilli is sufficient to produce tuberculosis within 3 months after injection into a healthy pig. Since the average number of such fecal masses passed during 24 hours is 60, a rough estimate of the degree of cage infectivity may be obtained. Nevertheless, in guinea pigs, variation of individual resistance to experimental tuberculosis is considerably greater than is generally supposed. Among very many animals injected in the course of the present study, it has been noted that guinea pigs of the same weight, age and from the same source, inoculated either subcutaneously or intraperitoneally with a fixed quantity of tubercle bacilli taken from the same culture have survived from 6 weeks to 8 months. Those dying after a short period have presented an acute disseminated tuberculosis; those surviving many months, a more chronic fibrotic disease accompanied in two instances with cavitation in the lungs. This individual variation in the span of life and the type of disease has doubtless been accompanied by individual variations in the degree of fecal elimination of tubercle bacilli.

With virulent tubercle bacilli contaminating the food of the normal cage mates, it is readily understood why the intestinal and mesenteric route of infection is so constantly present in instances of spontaneous tuberculosis. In several instances of spontaneous tuberculosis, the lesions were limited to the mesenteric lymph node and to the spleen or to the mesenteric lymph node only. The association in many instances of disease of the cervical as well as of the mesenteric lymph nodes is significant and recalls the bovine tuberculosis of childhood. In a large series of tuberculous guinea pigs injected intraperitoneally, the mesenteric lymph nodes with general dissemination were involved in 8 per cent. In no one instance was the mesenteric lesion particularly prominent. The cervical lymph nodes are even more rarely involved in an experimentally produced disseminated tuberculosis. Although tonsillar tuberculosis and secondary

cervical lymphatic tuberculosis is often found in swine exposed to the feces of tuberculous cattle, I was unable to observe any gross manifestation of involvement of the tonsils in guinea pigs with spontaneous tuberculosis. In studying the route of spontaneously acquired tuberculosis in a small series of guinea pigs exposed to patients suffering with pulmonary tuberculosis, Bartel and Spieler (16) report one instance of a microscopic tuberculous lesion in the pharyngeal wall, but none in the tonsils.

In those instances in which the normal cage mates were exposed to animals with open ulcerative lesions, the type of infection was the same as in instances in which the source of infection was from the feces only. It is probable that if the ulcerations are large enough and the exposure long enough as in the experiments of Römer, 100 per cent of exposed animals will acquire spontaneous tuberculosis.

In only one instance of spontaneous infection among the guinea pigs directly exposed to infected cage mates were the lungs and the tracheobronchial nodes involved with sufficient prominence to indicate that the source of infection was bronchiogenic. The bronchiogenic type of infection is occasionally seen in guinea pigs kept in separate cages but exposed to tuberculous animals in the same animal room. The disease in these instances is comparable to the tuberculosis of the lungs and the hilum lymph nodes in childhood.

The experiments and instances reported in this paper show that spontaneous tuberculosis in guinea pigs is a readily contracted disease. Guinea pigs kept in separate cages in a room with tuberculous animals for a period of more than 3 months should not be used for experimental purposes. If guinea pigs in which suspected material has been injected for diagnostic purposes are killed or die after a period of more than 3 months and tuberculous lesions are found, it is possible that the tuberculous infection was spontaneously contracted. It may be difficult to determine an instance of tuberculosis in a guinea pig as spontaneously acquired, or experimentally initiated if the local lesion at the site of inoculation has healed.

In spontaneous tuberculosis in guinea pigs there is a prominent involvement of either the mesenteric or the cervical lymph nodes or both, secondary to disease of the digestive tract; or of the lungs and

hilum nodes. In my experiments, the portal of entry has been more frequently the intestinal tract. In two instances intestinal ulcers were found in the ileum. These were macroscopic in size. In every instance of spontaneous infection, the intestinal tract in its entire length was carefully examined but it is possible that instances of small ulcerations were overlooked. Further, it is not unlikely that intestinal lesions heal in the course of the infection and are therefore not found at autopsy.

The mesenteric nodes are massively involved and lesions occur in the spleen and liver. The lesions in the spleen are apt to progress more slowly and with considerable fibrosis. Occasionally, the mesenteric lesion may so completely heal that an entire lymph node may become fibrous and lesions which appear in the spleen may be mild and of a chronic type. It is possible that this type of spontaneously acquired disease may go on to complete healing. Not infrequently a mesenteric node was found entirely fibrous and yellowish white, with no other evidence of disease elsewhere in the body. Tuberculin tests during life occasionally showed mild reactions, yet at autopsy, no evidence of disease other than a fibrotic node was found. Such instances were, of course, not included in our series as there was no evidence of the presence of tubercle bacilli either by culture or by animal inoculation. Healed lesions probably occur in guinea pigs exposed to tuberculous infection.

CONCLUSIONS.

1. Normal guinea pigs exposed to tuberculous cage mates infected intraperitoneally and with no cutaneous ulceration readily contract spontaneous tuberculosis.

2. The incidence of spontaneous tuberculosis increases with the intensity and with the duration of exposure.

3. Spontaneous tuberculosis acquired from infected cage mates has with few exceptions the characters of an infection which has entered by way of the digestive tract, disease of mesenteric and cervical lymph nodes being conspicuous.

4. Guinea pigs exposed to tuberculous animals in the same room but not in the same cage may acquire tuberculosis which has the characters of a bronchiogenic infection associated with lesions of the lungs and tracheobronchial lymph nodes.

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A SPECIFIC FLOCCULATION REACTION OCCURRING BETWEEN ALCOHOLIC EXTRACTS OF PNEUMOCOCCI AND ANTIPNEUMOCOCCUS SERUM.*

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INTRODUCTION.

The study of the antigenic properties of lipoidal substances extracted from antigenic material by alcohol, ether, or other fat solvents has included investigation of the significance of lipoidal constituents of the red blood cell in the formation of lysins and agglutinins, the action of alcoholic extracts of organs in the production of heterogenetic antibodies and their reaction with Forssman's antigen, and, finally, of the specific nature of such lipoidal fractions obtained from bacterial cells. Until recently, experimental work on the bacterial cells had been limited almost entirely to the immune reaction in tuberculosis. No attempt is made to review the large amount of work carried out in this particular field by numerous authors, as this has lately been done by Wadsworth, Maltaner, and Maltaner (1). In their study of the complement fixation reaction in tuberculosis, they report a systematic investigation into the distribution of the antigenic properties in tubercle bacillus cultures, which they found to be associated with the acetone-insoluble lipoids.

While the rôle of non-specific lipoids, extracted from various tissues, in the numerous flocculation reactions devised for the serodiagnosis of syphilis, has long been recognized, the action of bacterial lipoids in a specific flocculation reaction had not been made the subject of extensive research until 1924 when Dujarric de la Rivière and Roux

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(2) described flocculation occurring between alcoholic extracts of meningococci and antimeningococcus serum.[1] They found that the combination of antimeningococcus serum with watery emulsions of alcoholic extracts of meningococci, reinforced with tincture of benzoin, would result in macroscopically visible specific flocculation, which they considered to be immunologically different from either the agglutination or the complement fixation reaction. The authors suggest the value of this reaction in the standardization of antimeningococcus serum. More recently, they (3) have also included the gonococcus in their studies. Georgesco (4) has reported the application of the same method to the serodiagnosis of cerebrospinal meningitis and typhoid fever. Lately, Klopstock (5) has obtained very significant results with alcoholic extracts prepared from pure cultures of *Treponema pallidum* in the complement fixation reaction for syphilis.

While engaged in a study of this flocculation reaction with the meningococcus, during which it was possible to confirm Dujarric de la Rivière's results with serums and extracts prepared in this laboratory,[2] it was decided to investigate whether a flocculation reaction of similar character occurring under essentially the same conditions, could be obtained with alcoholic extracts of pneumococci and antipneumococcus serum. Experimental work on this subject was prompted, partly, by the desire to ascertain whether such a flocculation reaction between bacterial lipoids and immune serum was not only species- but also type-specific. It is unnecessary to emphasize particularly in this connection that, in comparison with the pneumococcus, the types of meningococci at present appear to be less sharply differentiated. In the foreground of interest, however, stood the

[1] An alcoholic extract of meningococci prepared by grinding meningococci with silica and subsequently extracting the bacteria at 37°C. with 96 per cent alcohol was proposed by I. R. Mörch in 1922 (reported by T. Madsen in Reports on Serological Investigations, League of Nations, Health Organization, 1923, 35) as a standard antigen for testing the potency of antimeningococcus serum. This extract, diluted with salt solution, was mixed with decreasing amounts of serum and the mixture incubated for 18 hours at 37°C. A specific precipitation reaction occurred, which was found to provide a basis for the estimation of the therapeutic potency of the serum.

[2] Dr. Dujarric de la Rivière of the Pasteur Institute in Paris kindly provided us with reagents for comparative work with the meningococcus.

study of the relation of this new immune reaction to the therapeutic potency of the immune serum. Obviously, this would meet with obstacles in the case of antimeningococcus serum since no satisfactory test to measure unmistakably the therapeutic potency of this serum has yet been described, and the substantiation of its efficacy by clinical data is extremely difficult and unreliable. This problem would appear to be more accessible to study with the pneumococcus, inasmuch as the standardization of antipneumococcus serum, based on the protection test in mice, reflects with a high degree of accuracy the therapeutic potency of this serum.

EXPERIMENTAL WORK.

Antigens.—The bacterial antigens were prepared by extracting the centrifugalized, washed sediment of 1500 cc. of an 18 hour pneumococcus broth culture in 40 cc. of absolute or 95 per cent ethyl alcohol for a period of 4 weeks, at room temperature. The extracts were then filtered through paper and were ready for use. Antigens obtained by extracting the bacterial sediment for 2 hours with boiling alcohol in a reflux condenser were found to be only slightly weaker in flocculating power than those prepared by the first method. The cultures used were: Three Type I strains—two standard strains, Pneumococcus Type I No. 5 (Neufeld) and Pneumococcus Type I No. 5D (U. S. Hygienic Laboratory), and a Pneumococcus Type I strain recently isolated in this laboratory from pneumonic sputum; one Type II strain and one Type III strain, both the latter isolated at and received from the Hospital of The Rockefeller Institute. The extracts used for the major part of the work described in this paper were prepared from Pneumococcus Type I No. 5.

It seemed of interest to obtain some information on the chemical and biological nature of the substances extracted by the alcohol.

5 cc. of extract, Type I, were evaporated, and the dry residue, 6 mg., was resuspended in 2 cc. of physiological salt solution, which produced an almost clear solution. It gave no biuret reaction, but reacted very slightly with Millon's reagent. A Molisch test resulted in a very faint purple color comparable to the one obtained with a 1:500,000 dextrose solution. When the solution was overlaid in 0.3 cc. amounts on 0.3 cc. of each of the tree types of antipneumococcus serum, in no instance did the solution give a precipitin reaction (incubation up to 3 hours at 37°C.). The extracted Type I organisms, on the other hand, suspended in salt solution, were still specifically agglutinated by Type I antipneumococcus serum, although they had become insoluble in bile. This suspension was boiled for a short time, to obtain some of the soluble substances in solution, filtered, and the clear filtrate tested for a precipitin reaction with antipneumococcus serums of

Types I, II, and III. A very distinct type-specific precipitation occurred. In order to obtain information on the antigenic properties *in vivo* of the substances extracted by the alcohol and of the bacterial cells after extraction, 20 cc. of alcoholic extract were evaporated, and the dry residue was resuspended in 15 cc. of salt solution. Simultaneously, the bacterial cells after extraction, representing the treated sediment of 750 cc. of broth culture, after several washings, were suspended in 15 cc. of salt solution. One rabbit was given the total volume of the first preparation divided into four subcutaneous injections; another rabbit, the total volume of the second preparation similarly divided. Both animals were bled on the 5th day following the last injection. While the serum from the rabbit which had received the emulsion of the residue of the alcoholic extract gave neither an agglutination nor a precipitin reaction, the serum of the rabbit treated with the bacterial cells after extraction with alcohol, agglutinated faintly up to a 1:2 dilution and gave, undiluted, a distinct precipitin ring with the filtrate of a 48 hour autolyzed pneumococcus broth culture. The two rabbits were then tested for the degree of immunity developed, by the intravenous injection of 0.001 cc. of a highly virulent pneumococcus culture which had been passed through rabbits for a number of years. This dose, which killed a normal control rabbit in 48 hours, killed the rabbit which had received the emulsion of the residue of the alcoholic extract in 72 hours, while the other animal, immunized with the alcohol-treated bacteria, survived. The same animal, when injected 4 days later with 0.1 cc. of the same culture, was fully protected.

Serums.—The serums used for the tests were monovalent antipneumococcus serums obtained from horses immunized by various methods. Incidentally, a small number of immune serums from rabbits immunized with the whole pneumococcus culture for various periods of time, were tested. Finally, it has been possible to examine several samples of serum from pneumonia convalescents, obtained through the courtesy of Dr. Cole from the Hospital of The Rockefeller Institute in New York City and from Dr. Ordway of the Albany Medical College.

The Test.—In setting up the test, Dujarric de la Rivière's original method was modified in many respects. The addition of an unspecific reinforcing agent to the specific alcoholic bacterial extracts proved to be indispensable in order to make the flocculation reaction macroscopically readily visible. A 10 per cent alcoholic extract of Sumatra benzoin[3] was used for this purpose after it had been found that lecithin and cholesterol, although capable of reinforcing the intensity of the reaction considerably, did not induce as rapid a flocculation as did the benzoin.

[3] It would seem advisable to use no benzoin tincture which is older than 2 months, since evidence of deterioration was noted after that time. The bacterial antigens, on the other hand, appear to be stable for a longer period.

The technique of the test as finally adopted is briefly as follows:

To one part of benzoin tincture are added twelve parts of alcoholic bacterial antigen. This initial mixture is suspended in 0.85 per cent salt solution by adding quickly 25 cc. of the salt solution to 0.4 cc. of the initial mixture. It is important to add the diluent very quickly. Experiments in which buffered salt solutions of different pH values were used in preparing this suspension, indicated a neutral medium as the most desirable for the reaction. The colloidal suspension thus obtained is slightly opaque and just on the verge of spontaneous flocculation; if left standing at room temperature or shaken vigorously, it will flocculate by itself. In order to insure conditions of maximum sensitivity, it is necessary to determine for each new antigen and new benzoin tincture the optimum balance between bacterial antigen, benzoin, and salt solution. A constant volume of 2.5 cc. of this suspension is mixed with varying amounts of serum, starting with 0.5 cc. and ending with 0.05 cc. It is not advisable to test smaller amounts than 0.05 cc. because it was found that less than 0.05 cc. of normal serum is insufficient to stabilize the suspension; the comparable range of specific flocculation is thus limited to the amounts indicated. The tubes are incubated, half immersed in a water bath at 40°C. since this temperature was found to provide conditions of the greatest sensitivity, maintaining at the same time the highest degree of specificity; it was observed that normal horse serum flocculated slightly above 46°C. Readings are made after ½ hour, 1 hour, and 2 hour intervals to determine the degree of flocculation. Occasionally, another reading is made on the following day.

Results.—It was found that a number of different antipneumococcus, Type I, Type II, and Type III serums, when tested in the manner described, gave a flocculation reaction of varying intensity with their homologous alcoholic bacterial antigens. While a great many of the fresh Type I serums gave a marked flocculation with Type I antigens, two older Type II serums and one older Type III serum, the only serums of these types available, reacted only faintly with their respective antigens. The species specificity of this reaction was controlled (1) by testing several normal horse serums as well as other immune serums, such as, antimeningococcus serum and antidysentery serum, with the pneumococcus antigen, (2) by testing alcoholic extracts of other microorganisms prepared, in a similar way, with antipneumococcus serum. In no case was flocculation observed under such conditions within the limits of the experiment.

In order to determine whether this reaction exhibited also type specificity, pneumococcus antigens, Types I, II, and III, were tested with the heterologous type serums. Some slight cross-reactions oc-

curred between Type II and Type III antigens and Type I serum as recorded in Table I, but no overlapping was noted with Type I antigen.

It was soon found that a number of Type I serums older than approximately 1 year, flocculated slightly, if at all, in spite of the fact that they still agglutinated and protected very well. It must remain undecided at present whether serums lose their flocculating power by aging, because no serum giving flocculation has yet been observed for longer than about 4 months. For the same reason, it is impossible to state whether the weak reactions obtained with two Type II serums and one Type III serum with their homologous antigens are attributable to the age of the serums, which were between 1 and $2\frac{1}{2}$ years old, or whether flocculation runs parallel to the protective action of antipneumococcus serum in general, having no relation to the agglutinative titer. As is usually the case, these Type II and Type III serums had very little, if any, protective action, while their agglutination titer was approximately the same as that of the Type I serums.

The results of these tests are tabulated in Table I.

A number of serums from rabbits immunized with pneumococcus, Type I, were tested for flocculation. While the reactions with these rabbit serums, which had a comparatively high agglutination titer, were very weak, the tests were too few to be of much significance. There is some reason to believe that, after prolonged immunization, the serum may acquire definite flocculating power.

The results obtained with the pneumonia convalescent serums are still too scanty to warrant a detailed report, although definite flocculation occurred in several instances with serum taken as early as the 2nd day of the disease. Occasionally, however, a very slight flocculation with the largest amount of normal human serum tested, 0.5 cc., was noted. In this connection, it should be recalled that Wadsworth (6), in his early work, obtained a precipitin reaction with high dilutions of convalescent pneumonia serums by using extracts of pneumococci. The centrifugalized fresh pneumococcus cells had first been shaken with hypertonic salt solution to increase the plasmoptysis, the extract then being made isotonic by the addition of water. He also observed a reaction with serums from apparently

healthy persons, in a dilution of 1:10, after 16 hours. It is hoped to improve the technique in future work so that significant results may be secured.

Further study was directed to a closer investigation of the nature of this flocculation reaction. It was found that supernatant serum which had flocculated, would not flocculate again, while the agglutination titer and the protective action of such a serum had remained practically unaffected; the solution of the flakes in salt solution up to the original volume released no protective action. Next, the effect of absorption was studied. It appeared that Pneumococcus Type I serum which, after absorption for 6 hours at room temperature with Type I organisms, had lost completely its agglutinative action, showed no flocculation whatsoever for the first 2 hours of incubation. Left overnight at room temperature, there was, however, considerable flocculation, although much less than occurred in the non-absorbed serum even after 2 hours' incubation. Later, the effect of heating for $\frac{1}{2}$ hour and 1 hour at 56°C. was studied in two different Type I serums. The results obtained did not agree. While heating for $\frac{1}{2}$ hour impaired the flocculating power of one serum considerably and heating for 1 hour completely destroyed it, the other serum, after being heated for $\frac{1}{2}$ hour, flocculated almost as well as the non-heated serum and even heating for 1 hour did not destroy its flocculating power. The agglutinative action of these two serums was similarly affected by heating as was the flocculating power. The addition of complement, in the form of fresh normal horse or guinea pig serum, did not reactivate the heated serum nor did the addition of complement to old serums improve their flocculating power.

Finally, the relation of this flocculation test to the therapeutic potency of Type I antipneumococcus serum was studied. Over forty serums obtained from ten different horses at various stages during immunization were tested for flocculation, and the results of these tests were compared with the protective titer ascertained by the routine method of standardization in mice. Although it had already been observed that the serums in repeated tests exhibited approximately the same flocculating power on different days, a serum which flocculated well was chosen as a standard and run along simultaneously with normal horse serum with each test, as a control. The results are tabulated in Table II.

TABLE I.

*Experiments on Type and Species Specificity of Flocculation Reactions with Alcoholic Extracts of Pneumococci.**

Pneumococcus Type I antigen										Pneumococcus Type II antigen									
Serums	Date bled	Mar. 9 0.5 cc. of serum incubated for			Mar. 9 0.3 cc. of serum incubated for			Mar. 9 0.1 cc. of serum incubated for			Serums	Date bled	Mar. 9 0.5 cc. of serum incubated for			Mar. 9 0.3 cc. of serum incubated for			Mar. 9 0.1 cc. of serum incubated for		
		½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.			½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.
Pnc. Type I Serum 312	1.7.26	2+	3+	4+	2+	2+	3+	±	1+	1+	Type I Serum 312	1.7.26	−	±	1+	−	±	±	−	−	−
" " I " 313	1.26.26	2+	3+	4+	2+	3+	4+	2+	3+	3+	" I " 313	1.26.26	−	±	1+	−	±	±	−	−	−
" " II " 93	10.22.23	−	−	−	−	−	−	−	−	−	" II " 93	10.22.23	−	±	1+	−	±	±	−	−	−
" " II " 85	7.16.23	−	−	−	−	−	−	−	−	−	" II " 85	7.16.23	−	±	±	−	−	−	−	−	−
" " III " 62	1.4.25	−	−	−	−	−	−	−	−	−	" III " 62	1.4.25	−	−	−	−	−	−	−	−	−
Normal Horse Serum (H. 144)	4.24.25	−	−	−	−	−	−	−	−	−	Normal Horse Serum (H.144)	4.24.25	−	−	−	−	−	−	−	−	−
" " " (H. 140)	2.5.24	−	−	−	−	−	−	−	−	−											
" " " (H. 140)	2.2.26	−	−	−	−	−	−	−	−	−											
Antimeningococcus Serum 145	3.3.25	−	−	−	−	−	−	−	−	−											
Antidysentery Serum 75	2.7.25	−	−	−	−	−	−	−	−	−											

Pneumococcus Type III antigen										Antigens from other microorganisms tested with Type I Antipneumococcus Serum 312									
Serums	Date bled	Mar. 9 0.5 cc. of serum incubated for			Mar. 9 0.3 cc. of serum incubated for			Mar. 9 0.1 cc. of serum incubated for			Antigens	Mar. 10 0.5 cc. of serum incubated for			Mar. 10 0.3 cc. of serum incubated for			Mar. 10 0.1 cc. of serum incubated for		
		½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.		½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.
Type I Serum 312	1.7.26	—	—	±	—	—	—	—	—	—	*Streptococcus viridans*	—	—	—	—	—	—	—	—	—
" I " 313	1.26.26	—	—	1+	—	—	—	—	—	—										
											Micrococcus catarrhalis	—	—	—	—	—	—	—	—	—
" II " 93	10.22.23	—	—	—	—	—	—	—	—	—										
" II " 85	7.16.23	—	—	—	—	—	—	—	—	—	Meningococcus 10-B	—	—	—	—	—	—	—	—	—
											" W 30-B	—	—	—	—	—	—	—	—	—
" III " 62	1.4.25	±	1+	1+	±	1+	1+	—	—	—	" 46-B	—	—	—	—	—	—	—	—	—
											" 79-B	—	—	—	—	—	—	—	—	—
Normal Horse Serum (H. 144)	4.24.25	—	—	—	—	—	—	—	—	—										
											Alcohol	—	—	—	—	—	—	—	—	—

* The intensity of flocculation is designated as follows:

— = diffuse opacity.
Tr. = trace (faintest granulation).
± = slight granulation.
1+ = small flakes.
2+ = medium sized flakes.
3+ = large flakes.
4+ = heavy flocculent precipitate at the bottom leaving supernatant fluid clear.

TABLE II.

Comparison between Flocculation Reaction, Agglutination Titer, and Protective Action of Different Type I Antipneumococcus Serums.

Horse and bleeding Nos.	Serum No.	Immunization	Date bled	Dates of flocculation tests 1926	Flocculation after 1 hr.'s incubation at 40°C.			Date of agglutination test	Agglutination after 2 hrs.' incubation at 37°C.				Date of protection test	Protective action	
					0.5 cc.	0.3 cc.	0.1 cc.		1:10	1:20	1:40	1:80		Standard serum	Test serum
														0.2 cc. protected	
														Culture	
														cc.	cc.
143–10	271	11 mos.	7.24.24	2.5 2.9	Tr.	Tr.	–	9.11.24	3+	3+	–	–	9.17.24	0.1	0.2
–11	273	11 "	7.29.24	2.5 2.9	2+	2+	±						9.17.24	0.1	0.2
–12	279	1 yr. 3 mos.	1.3.25	2.5 2.9 2.11	Tr.	Tr.	Tr.						1.7.25	0.1	0.3
152–13	270	1 yr.	7.16.24	2.5 2.9	–	–	–	9.11.24	2+	–	–	–	7.31.24	0.1	0.1
–14	272	1 "	7.29.24	2.5 2.9 2.11	±	Tr.	Tr.						9.17.24	0.1	0.2
190–3	289	4 mos.	4.22.25	1.29 2.5	±	–	–	2.1.26	4+	2+	–	–	5.13.25	0.1	0.1*
189–4	290	5 "	5.6.25	1.29 2.5	1+	Tr.	Tr.	7.17.25	3+	3+	1+	1+	5.28.25	0.2	0.1
193–3	303	105 days	11.7.25	2.21 2.24	±	–	–	3.18.26	3+	2+	1+	±	11.11.25	0.1	0.1*
–4	309	136 "	12.8.25	2.21 2.24	3+	2+	1+	2.1.26	3+	3+	2+	–	12.9.25	0.1	0.2
–5	310	142 "	12.14.25	2.21 2.24	3+	2+	2+	3.18.26	3+	3+	3+	2+	12.16.25	0.1	0.1
–6	311	164 "	1.5.26	2.21 2.24	3+	3+	2+	3.18.26	3+	3+	3+	1+	1.6.26	0.1	0.1
–7	313	185 "	1.26.26	2.21 2.24	3+	3+	3+	3.18.26	2+	3+	3+	–	1.27.26	0.1	0.1
–8	316	207 "	2.17.26	2.21 2.24	2+	2+	1+	3.18.26	3+	3+	2+	–	3.4.26	0.1	0.1
–9	318	221 "	3.2.26	3.5 3.7	2+	1+	1+	3.18.26	3+	3+	2+	–	3.16.26	0.2	0.2
161–3	302	105 "	11.7.25	2.11 2.24	2+	1+	–	3.18.26	3+	2+	±	–	11.11.25	0.1	0.1*
–4	307	135 "	12.7.25	2.11 2.24	1+	Tr.	–	3.18.26	3+	3+	1+	–	12.9.25	0.1	0.1**

−5	312	166 days	1.7.26	2.11	2.24	3+	2+	1+	3.18.26	3+	2+	2+	−	1.13.26	0.1	0.1
−6	314	188 "	1.29.26	2.11	2.24	3+	3+	1+	3.18.26	3+	3+	1+	−	2.10.26	0.1	0.1
−7	317	215 "	2.20.26	3.5	3.17	1+	±	±	3.18.26	3+	3+	−	−	3.4.26	0.1	0.2
−8	322	231 "	3.13.26	3.17	3.19	±	±	−	3.18.26	3+	3+	−	−	3.16.26	0.2	0.1
−9	323	244 "	3.27.26	4.9		−	−	−	4.28.26	3+	2+	−	−	3.31.26	0.1	0.1*
−10	327	263 "	4.16.26	4.20		±	±	−	4.28.26	4+	−	−	−	4.21.26	0.1	0.1
201–1	306	111 "	11.13.25	2.5	2.9	1+	±	−	3.18.26	3+	1+	±	−	11.18.25	0.2	0.1
−2	308	136 "	12.8.25	2.5	2.9	2+	1+	±	2.1.26	3+	−	−	−	12.9.25	0.1	0.1
207	Norm. bl.	0	12.19.25	2.23	2.26	−	−	−								
	1st trial bl.	38 "	1.26.26	2.23	2.26	−	−	−						2.3.26	0.3	0.1**
	2nd " "	56 "	2.13.26	2.23	2.26	−	−	−	3.18.26	3+	1+	−	−	2.17.26	0.1	0.1**
−1	320	82 "	3.11.26	3.15	3.17	1+	±	−	3.18.26	3+	3+	±	−	3.16.26	0.2	0.1
−2	325	103 "	4.1.26	4.9		−	−	−	4.28.26	3+	3+	1+	−	4.14.26	0.1	0.2
−3	328	124 "	4.22.26	4.22		1+	1+	±	4.28.26	4+	2+	−	−	4.26.26	0.1	0.2
−4	331	147 "	5.13.26	6.1		1+	1+	1+	5.19.26	3+	3+	−	−	5.19.26	0.2	0.2
150	Norm. bl.	0	11.27.25	2.23	2.26	−	−	−								
	3rd trial bl.	64 "	1.30.26	2.23	2.26	−	−	−	4.28.26	3+	1+	−	−	2.3.26	0.2	0.1
	4th " "	73 "	2.8.26	2.23	2.26	−	−	−	3.18.26	3+	3+	−	−	2.10.26	0.2	0.1
−1	315	82 "	2.17.26	2.23	2.26	Tr.	−	−	3.18.26	3+	2+	1+	−	2.24.26	0.1	0.1
−2	319	96 "	3.3.26	3.15	3.17	±	−	−	3.18.26	3+	2+	±	−	3.10.26	0.1	0.1
−3	324	124 "	3.31.26	4.9		2+	2+	1+	4.28.26	3+	3+	±	−	11.15.26	0.1	0.1†
151	Norm. bl.	0	12.19.26	3.5	3.15	−	−	−								
	1st trial bl.	38 "	1.26.26	3.5	3.15	−	−	−						1.27.26	0.1	0.1**
	2nd " "	56 "	2.13.26	3.5	3.15	−	−	−	3.18.26	3+	−	−	−	2.17.26	0.1	0.1**
−1	321	82 "	3.11.26	3.15	3.17	1+	±	−	3.18.26	3+	2+	1+	−	3.16.26	0.2	0.2
−2	326	103 "	4.1.26	4.9		−	−	−	4.28.26	4+	1+	−	−	4.21.26	0.1	0.1
−3	329	124 "	4.22.26	4.29		−	−	−	4.28.26	4+	2+	−	−	4.26.26	0.1	0.1
−4	330	147 "	5.13.26	6.1		±	±	±	5.19.26	4+	2+	±	−	5.19.26	0.2	0.2

* Protected partially. ** Did not protect.

† Earlier tests of the serum, while irregular, suggested its value to be approximately equal to that of the standard serum.

In studying Table II, Horses 193 and 161 attract particular attention, inasmuch as it had been possible to follow a number of bleedings taken between the 3rd and the 8th or 9th month of immunization, and to compare the results of the protection test in mice and the agglutination titer with the flocculating power as measured by the test described. A relationship between the flocculating power of the serum from these horses and the protective action is suggested. In the case of Horse 161, a marked decrease in the flocculating power of the serum occurred before the end of immunization. This horse was in very poor physical condition when bled out. It is also of special interest to note that Horse 193 developed endocarditis toward the end of immunization, which condition apparently did not affect the protection titer of the serum, but there was a slight drop in the flocculating power. Horses undergoing active immunization by the intravenous inoculation of living virulent pneumococci frequently develop lesions in the heart valves, as first described by Wadsworth (7), in 1919, and yet their serums possess high protective action in mice against virulent pneumococci. In Horse 150, the flocculating power developed very much more slowly than the protective action and, in Horses 151 and 207, even at the end of the 5th month of immunization, there was only slight flocculation, although three previous bleedings of Horse 151 and two previous bleedings of Horse 207 had given a protection value at least equal to that of the standard serum. The agglutinative activity, as evidenced especially by Horses 207, 150, 151, appears much earlier than either the protective antibodies or the flocculating power, after which they run a somewhat parallel course (Horses 193 and 161). It is apparent that, in spite of frequent general agreement between flocculating power and protective action, there remain considerable discrepancies among individual serums which will have to be elucidated by further studies.

DISCUSSION.

Although, at present, it is impossible to give a lucid explanation of the mechanism of the reaction described, it would seem that an immune reaction, exhibiting strict species and a high degree of type specificity may be obtained with substances extracted from the pneumococcus cell by absolute or 95 per cent ethyl alcohol. The method

of preparing the alcoholic antigen does not preclude the possibility that, besides lipoidal substances, certain impurities of protein or carbohydrate character may have been carried over into the extract. The extracted material so far has been obtained only in such small amounts that an exact chemical analysis has not been possible; also, no other solvents have been used. It would seem, however, from the tests done that only traces of substances of protein or carbohydrate nature can have been present in the extract. The reaction apparently depends essentially upon the presence of bacterial lipoids.

In this connection, there comes to mind the work of Avery and Heidelberger (8–10), who, basing their conclusions upon precipitin tests, attribute the type specificity of the pneumococcus to a substance of carbohydrate nature while a nucleoprotein is designated as the carrier of the species specificity of the organism. On the other hand, Zinsser and his associates (11–14) have prepared, from various bacteria, including also the pneumococcus, substances—residue antigens—which are free from coagulable protein and are capable of reacting specifically in a precipitin test with the homologous antiserum. Both of these substances, the polysaccharide of Avery and Heidelberger and the residue antigen of Zinsser, were found by the respective authors to be incapable of producing antibodies. Perlzweig and Steffen (15), in studying different fractions of the pneumococcus for their immunizing effect in mice, found the alcohol-soluble fraction of a trypsin digest of Pneumococcus Type I organisms as active as the ordinary vaccine of heat-killed pneumococci in affording protection, while they failed to obtain immunity with alcohol, ether, chloroform, and acetone extracts made from intact, dried pneumococci. They also state that among the various antigenic solutions which were tested for a precipitation with immune serums, some of the potent alcohol-soluble immunizing antigens failed to react, while, in other instances, precipitation was observed. In this connection, the experiments of Brotzu (16), who found ether-extracted pneumococci to lack antigenic activity in the immunization of rabbits, are of interest.

It remains for further study to coordinate the results obtained with the alcohol-soluble substances described in this paper with those described by the investigators cited above.

Although the type specificity of the reactions is quite marked in some of the tests, more particularly with Type I antigen; in others, it is not quite so definite. Similarly, although the results of the flocculation reactions in general appeared to correspond to those of the protection test, in many instances they were divergent. Since the protection test varies so markedly with the virulence of the

pneumococcus, it is not possible to determine as yet which test yields the more uniform quantitative results. The extent to which differences in the activity of the several antigens and serums figure in the test and whether or not the test can be more accurately standardized for quantitative determination, will require special investigation. Nevertheless, the results obtained so far indicate the desirability of further study of this reaction in connection with the type diagnosis of pneumonia and suggest its value in the standardization of Type I antipneumococcus serum.

SUMMARY AND CONCLUSIONS.

1. A flocculation reaction has been described which occurs between alcoholic extracts of pneumococci and antipneumococcus serum.

2. The reaction appears to be species-specific. It is not strictly type-specific, as slight or moderate cross-reactions occurred between Type I serums and Type II and Type III extracts.

3. The flocculating power of the serum from five horses undergoing immunization with pneumococcus, Type I, did not develop to any extent before the end of the 4th or 5th month.

4. In the case of two of these horses in which it was possible to carry out parallel tests on a larger number of subsequent bleedings until the end of immunization, some relationship was suggested between the flocculating power and the protective titer as ascertained by the routine method of standardization in mice.

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AN EXPERIMENTAL STUDY OF GYE'S CANCER THEORY.

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The experiments of Gye (1) on the etiology of malignant growth, based on a study of the Rous chicken sarcoma, have attracted much attention. His conclusions offer an explanation not only of the cancer problem, but also, indirectly, of that of the nature of other types of filterable viruses. A repetition of his work has been undertaken in a number of different laboratories. At least four reports of such work have already reached the literature,—those of Murphy (2), Harkins, Schamberg, Kolmer, and Kast (3), Flu (4), and Cori (5). The results published in these papers differ widely among themselves, and all are at variance with those of Gye.

Gye believes that tumors in general are caused by a filterable virus probably common to all tumors irrespective of variety or source and capable of growth *in vitro*. Under experimental conditions, this virus is unable to infect without the aid of another factor which ruptures the defense of the cell. In the case of filterable chicken tumors, such an agent is present, which is readily separable from the cells. He calls this the "specific factor," since it is effective only in chickens to produce always the same type of tumor from which it is derived.

These conclusions are based, primarily, on two types of evidence. In the first place, Rous sarcoma filtrates, when centrifuged for an hour at 9000 revolutions per minute, are separated into a sediment and a supernatant fluid, neither of which, alone, appears to be capable of producing a tumor in chickens, but, when injected together, will do so. Gye admits that it is difficult to get clear-cut results with this technique, and Flu, who is the only one to have published his experiences in attempting to repeat this phase of the work, obtained one positive result out of three experiments, but feels that it is unwarranted to draw definite conclusions from this without further controls. The second and main line of

proof is more difficult to carry out, and subject to more theoretical and practical criticism. Specific factor from the Rous sarcoma is obtained by treating the filtrate with chloroform under definite conditions, which is supposed to kill the virus. Preparations of the latter are supplied by cultures of the Rous sarcoma or of various rat and mouse tumors, and, in one case, of a human mammary carcinoma. In all of Gye's published protocols the injection of either factor alone, in a quantity of 1.0 cc. into a chicken, gave no result, while a mixture of 0.5 cc. of each always produced a large tumor in 2 weeks or less.

Gye's primary tumor cultures were made by adding 1 gm. of the tumor to 5 cc. of Hartley's broth plus 0.2 per cent KCl, to which was added 1.0 cc. of rabbit serum. Subcultures were made by transferring a loopful of a primary culture, after 4 days' anaerobic cultivation at 36° in a McIntosh and Fildes jar, to similar media containing a fragment of 10 to 14 day chick embryo. Subcultures of the Rous sarcoma as far as the twelfth generation were found to be active, while only primary cultures of the mammalian tumors were used.

Gye does not include protocols in his paper in which uninoculated tubes of Hartley's broth containing rabbit serum and embryonic tissue are used as controls after incubation. Murphy (2) reports on three short experiments in which successful reactivation of the chloroformed filtrates by means of tumor cultures was shown and in which reactivation with such uninoculated controls was also positive, and believes the results are due to an acceleration of a partially destroyed agent by means of some substance common to rapidly growing tissues such as tumor, embryo, or placenta.

Cori (5) reports one similar experiment out of eleven, the other ten being negative. Flu (4) carries Murphy's criticism a step further and reports successful reactivation not only with tumor cultures and embryo "cultures," but also with similarly prepared cultures of liver and kidney tissues from both chickens and guinea pigs.

On the other hand, Harkins, Schamberg, Kolmer, and Kast (3) obtain different results. If enough chloroform is added to the Rous sarcoma filtrates so that tumors are not produced by the injection into chickens of this substance alone, the addition of tumor cultures made according to Gye's methods does not restore infectiousness.

It should be stated at this time that the results to be described in this paper are in accord with those of Harkins, Schamberg, Kolmer, and Kast. Before entering into a consideration of the possible reasons for the divergence of results in different laboratories our experiments may be presented.

Methods.

The writer wishes to acknowledge with thanks the assistance obtained in several letters from Dr. Gye, and in conferences with Dr. Max Cutler. It has been our aim to follow Gye's directions in attempting to repeat his experiments. These are commented upon only where necessary.

The Tumor.—Dried tumor of the Rockefeller chicken tumor No. 1 (Rous sarcoma), marked "Desiccation 31, VII, 1, 25" was kindly supplied us by Dr. Rous. Injection of a small quantity of this, suspended in Ringer solution, into the breast muscle of chickens led to the production of tumors in about 14 days, with death in 3 to 4 weeks. From such tumors, inoculations of cells, desiccated or glycerolated tissue or filtrates have uniformly produced tumors in 1 to 2 weeks. Metastases have been frequent, but not invariable, to liver, heart muscle, and lungs. In many cases they have not been looked for, since the local tumor has been the important point in the work. Microscopic examination has also not been made of all tumors, but when done, the picture agreed with the earlier published descriptions of the tumor. We have had little difficulty with contaminated cultures. Instead of soaking the chicken in lysol, before excising tumor tissue for culture, we have found it more convenient, and equally satisfactory, to kill the chicken quickly, dip for a moment in boiling water, strip off the feathers for some distance around the site of the tumor, and paint the skin with strong iodine.

Gye and Andrewes (6) have published the interesting observation that the Rous sarcoma occasionally undergoes a change in properties becoming "non-filterable." The tumors we have used have not invariably been tested, but they have at intervals passed through medium Berkefeld filters (no more failures than one would expect in this type of experiment), and have survived glycerolation for 3 days or longer.

The Chickens.—Most of the experiments have been carried out on pure bred Barred Rock chicks of 6 to 8 weeks of age. While the tumor usually appears in 10 days, there have been instances of much later development, and to guard against overlooking tumors, the chickens have been kept for 3 months after inoculation, or until death from intercurrent disease occurred.

Sand Filtrates.—Made exactly as described by Gye and Andrewes (6) and filtered through sand and paper pulp in an apparatus similar to his. It has usually been necessary to use gentle suction in order to get the material through in a reasonable length of time, and the Ringer suspension has occasionally been centrifuged for a short time to hasten subsequent filtration. The sand filtrates have, with a single exception, proved to be highly infectious, producing rapidly growing tumors in quantities of from 0.1 cc. to 0.01 cc. or even less.

While the proportionality which Gye stresses between quantity injected and size and rate of growth of tumor holds to a certain extent, it has not impressed us as being sufficiently striking to serve as the basis for a theory that a chemical substance and not a living agent is involved. In point of fact, while Gye bases the reasoning for his experiments upon this observation, he nevertheless always uses a quantity of virus culture equal in amount to the chemical specific factor, whereas, if his hypothesis holds, a trace should be sufficient.

After a number of preliminary experiments in which ordinary chloroform was used, Merck's reagent chloroform was redistilled, the middle third saved and kept

in a dark bottle in the ice box, in order to avoid the action of possible impurities on the filtrate. It was found that tumor filtrates varied considerably in their resistance to the action of the reagent. A number of experiments were wasted in attempting with a single quantity of chloroform to produce a suitable specific factor. The results showed almost uniformly either a development of tumor with both specific factor control and mixture, or no tumor with either. Gye states that he uses "a few drops" of chloroform, sufficient to saturate, but not a large excess. We found that filtrates could be saturated, as shown by the presence of droplets of the reagent at the bottom of the tube after mixing, and still be infectious after 3 hours' incubation at 37°C. A larger excess led to complete inactivation so that it was not possible to reactivate by any virus tried and, as stated above, the amounts necessary to bring about these results varied from one tumor to the next

In order to avoid this variation, desiccated tumor material was used for the preparation of some of the chloroform filtrates, since it must contain both "specific factor" and "virus." By preparing a quantity of this from a single large tumor, it should be possible to have uniform material for a number of experiments. Three or four different portions of the same sand filtrate were used in each experiment, and varying amounts of chloroform measured in with a fine, graduated pipette instead of being added in drops. It was noted that where Gye's directions for adding the chloroform were followed, *i.e.*, allowing it to run down the side of the tube so that a portion floated on the surface, followed by incubation at 37° for half an hour before mixing, most of that which floated was lost by evaporation, and the quantity subsequently mixed with the filtrate was less, and in the case of quantities as small as 0.05 cc., markedly less than it was intended to add. In some of the experiments we therefore mixed the chloroform with the fluid immediately after adding by means of drawing up repeatedly in a capillary pipette, and again mixed at the end of 30 minutes in the same way.

At the end of 3 hours the chloroform was removed by suction, by means of an oil pump. A great deal of frothing always took place, which made it necessary to transfer the fluid to a suction flask before carrying out this step, and to apply the suction carefully for some minutes. After a time the frothing ceased, and the full force of the pump could be used to obtain violent boiling. In transferring the material to the suction flask, the greater part of the excess chloroform usually remained adhering to the test-tube as an emulsion.

The protocols of the experiments in which desiccated tumor tissue was used will be given later, together with a discussion of the results. Since in general the experiments were not satisfactory, further work was done, following the same plan by again using filtrates from fresh tumors.

Virus Preparations.—Several different lots of Hartley broth were used in the course of the work. Beef muscle, ox heart, and horse muscle were all tried, but no difference was apparent. Gye, in a personal communication, asserts that not all rabbit sera are equally good, and that it is impossible to tell, except by trial,

which ones are suitable. He says, further, that in carrying a strain of the virus along in culture, at some stage the organism may die out, producing what he calls a "stop." Further subcultures from such a tube always produce negative results. Primary cultures of either chicken or mammalian tumors, he says, do not require rabbit serum, but subcultures will never succeed without it. These facts make it extremely difficult to know whether negative results may not be due to faulty cultures, and there seems to be absolutely no way to control them. In the early stages of the work "passage strains" of the Rous sarcoma were used. It was readily shown that primary cultures were infectious, and aerobically grown primary cultures which Gye occasionally used as virus (oxidation, according to his theory, destroying the specific factor rather quickly) usually gave positive controls in our hands. Upon receiving the above details as to cultures from Dr. Gye, we changed the character of our cultures, omitted the rabbit sera, and used only primary cultures of the Jensen rat sarcoma, or some other transplantable rat or mouse tumor. Controls containing chick embryonic tissue or rat or mouse embryo and placenta have been frequently used.

After 48 hours' anaerobic incubation, the jar is opened and the tubes examined grossly and by streaking out on blood agar, for contaminations. They are then returned to the jar and incubated 2 days longer before use. At the time of using, any contaminated tubes, as shown by the blood agar cultures, are discarded, the remainder are examined by stained smear. Several tubes of each type of culture are always made and before use the uncontaminated tubes of the same material are mixed together. This would seem to be the surest way of getting a serviceable virus preparation. In some cases the jar has not been opened at the end of 2 days, but inspection and stained smear at the time of use have been relied upon. Cultures made on blood agar plates at the time of using, as a check on the smear, usually, but not invariably remain sterile. There is, in general, little difficulty about contamination when the ordinary precautions of bacteriological technique are followed. If, however, a tumor is used which has begun to ulcerate, practically every tube is unavoidably contaminated.

Injections and Method of Control.—A small amount of sterile Kieselguhr is added to each preparation and the tube shaken before drawing up the material into the syringe. Where mixtures of specific factor and virus are made, the solutions are pipetted separately into a clean tube, the greatest care being taken not to contaminate one tube with the contents of another. Injections have been made into the muscles of the breast, legs, and upper wing.

The question of a suitable method of control is a most puzzling one, and at the same time, is of the utmost importance. There is a certain amount of difference in susceptibility to small quantities of the agent even in young Barred Rock chicks. Hence, one can never be sure that a negative control with 1.0 cc. in one chick, and a take with 0.5 cc. in another chick is significant unless it happens consistently. Beside the individual resistance, there is some other factor which the writer is unable to define which obscures the matter. For example, the same

quantity of material of reduced infectivity injected into both sides of the breast of a number of chickens may give in one chicken no takes, in another takes on both sides, and, what is still more important, in still others *takes on one side and not on the other*. Probably some such element as the quantity of Kieselguhr, the degree of trauma, the relation of the injected material to large vessels or lymphatics, or the amount of hemorrhage, all factors beyond control, is the determining point. This statement will be illustrated in later protocols. Now, if controls are done in separate chickens, considerable numbers must be done or they are without significance. If they are done in the same chickens, the work is open to theoretical criticism for this reason: Granting that two substances, A and B, are necessary to produce the lesion, if A is injected at one site, B at another, and A and B at a third, it is conceivable that A or B may not remain localized, but may enter the circulation, be carried to another site, and finding there B or A substance, produce a false positive control. One has the choice of two evils. Probably the only safe procedure is to use both types of controls, and do the experiment on a large scale, so that numbers will offset individual and chance variations.

Criteria for Judging Results.—From what has been said, it is evident that one cannot expect every experiment of this type to be satisfactory. It is equally evident, if the theory is to hold, that in a single experiment the results should be consistent. That is to say, if thirty or forty chickens are done, many of them duplicates or triplicates, it should be evident in viewing the protocols that the same thing is taking place throughout that particular experiment. It is entirely without significance to pick out two or three chickens and, neglecting the others, accept a probably fortuitous result as an experimental fact. It is true that there may be a perfectly reasonable difference of opinion as to the significance of such an experiment, but one should have the opportunity of drawing his own conclusions from a complete protocol. We do not mean, necessarily, that every chicken used should be accounted for, although by doing this very thing, Harkins and his collaborators have presented a valuable piece of work, but within a given experiment, at least, all should be described, and where only a few chickens are used enough similar experiments should be done and described to show whether the result is an accident or not.

This point of view has been reached after using a large number of chickens in experiments of this type. Many of these, in the earlier part of the work, where only a few were used at a time, need not be presented here, since they were frankly negative in results, either from the use of too much or too little chloroform. Commencing with the work with desiccated tumor in which more chickens were used, each experiment will be summarized. Unfortunately, an epidemic of coccidiosis killed a number of the chicks in the experiment of May 28 and it has been impossible to completely eliminate the infection from the flock. Evidence of it has appeared in all subsequent experiments as occasional deaths with extreme emaciation.

Experiment April 14, 1926.—Desiccated tumor was used as a source of specific

factor. A large rapidly growing tumor was excised, from which 60 gm. of fairly healthy tissue was obtained. This was minced and frozen. It was then transferred to a desiccator containing P_2O_5, the air was exhausted with an oil pump, and the desiccator then placed in an ice box until the material was completely dry. It was then ground, and preserved over P_2O_5 in the dark. 0.75 gm. of this dried material, considered to be equivalent to about 5.0 gm. fresh tumor, was ground with sand and 100 cc. Ringer solution, filtered through a sand filter, and chloroformed in the usual manner. Three quantities of 20 cc. each were prepared, 0.1 cc., 0.6 cc., and 1.0 cc. respectively of chloroform being used, and the preparations designated as Cl_1, Cl_2, and Cl_3. In this preparation Gye's directions for chloroforming were followed. The virus used was a mixture of several tubes of third generation Rous sarcoma culture, containing chick embryo but no serum, because of a misconception as to the permissibility of omitting serum not only from primary cultures but from passage tubes.

Chick No.	Injection	Date of death or killing and autopsy finding	
1	1 Cl_1 — 1.5 cc.	5/12	—
	2 Cl_1 — 1.5 cc. + V — 0.5 cc.		+
2	1 Cl_1 — 1.0 cc.	6/1†	—
	2 Cl_1 — 1.0 cc. + V — 0.5 cc.		—
3	1 Cl_1 — 0.5 cc.	5/17	—
	2 Cl_1 — 0.5 cc. + V — 0.5 cc.		+
4	1 Cl_1 — 0.1 cc.	7/17	—
	2 Cl_1 — 0.1 cc. + V — 0.5 cc.		—
5	1 Cl_1 — 0.05 cc.	6/5†	+
	2 Cl_1 — 0.05 cc. + V — 0.5 cc.		—

Chicks 6 to 17—injected according to same protocol with Cl_2 and Cl_3 V. No tumor resulted in any of them.

† Chickens marked thus died on the date shown either from the tumor or from intercurrent disease, mostly extreme emaciation (following coccidiosis?) with occasional instances of chicken paralysis and undetermined causes. Where the symbol is omitted, and it is not stated otherwise, it is signified that the chicken was killed on that date or missing without record subsequently.

The tumors developing at the site of the injection of the mixture in Chicks 1 and 3 are completely offset by the one found in Chick 5

at the site of injection of chloroform filtrate alone in a quantity of only 0.05 cc. and illustrate our main point that not only variation in chickens but some uncontrollable variation at the site of injection itself may determine formation of a tumor. It will be noted that since no serum was added to these virus cultures, no living virus should have been present in these tubes, and consequently, no accelerative action expected.

Experiment May 12, 1926.—Sand filtrate prepared from desiccated tumor. Eight tubes of 10 cc. each divided into four pairs, and chloroform added to each tube, as follows: 1st pair, 0.02 cc. each, 2nd, 0.03, 3rd, 0.04, and 4th, 0.05 cc. Water bath half an hour, mixed with capillary, and water bath 2½ hours longer. Each pair of similar tubes was mixed and the chloroform boiled out *in vacuo*. It was noted at time of mixing with capillary that all of the chloroform added to one tube of the first pair had evaporated from the surface, the quantity being too small to form a drop of sufficient size to sink. The preparations are as usual marked Cl_1, Cl_2, Cl_3, and Cl_4.

Virus preparations are as follows:

V_1—4 day primary anaerobic Mouse Sarcoma 180 sterile by smear, but shown later by culture to be contaminated.

V_2—4 day primary anaerobic Rat Sarcoma 10 sterile by smear and culture.

V_3—Uninoculated tube of 10 day chick embryo in Hartley broth, 4 days' anaerobic incubation, sterile.

Chick No.	Injection	Date of death or killing	Result 1	Result 2
18	1 Cl_1 — 1.5 cc.	6/13†	++	
	2 Cl_1 — 1.5 cc. V_1 — 0.5 cc.		±	
19	1 Cl_1 — 1.0 cc.	6/10	++	
	2 Cl_1 — 1.0 cc. V_1 — 0.5 cc.		+	
20	1 Cl_1 — 0.5 cc.	6/21†	++	
	2 Cl_1 — 0. 5 cc. V_1 — 0.5 cc.		+	
21	1 Cl_1 — 1.5 cc.	6/7	+	
	2 Cl_1 — 1.5 cc. V_2 — 0.5 cc.		+	
22	1 Cl_1 — 1.0 cc.	7/23	−	
	2 Cl_1 — 1.0 cc. V_2 — 0.5 cc.		−	
23	1 Cl_1 — 0.5 cc.	6/15†	++	
	2 Cl_1 — 0.5 cc. V_2 — 0.5 cc.		+	
24	1 Cl_1 — 1.5 cc.	6/5	++	
	2 Cl_1 — 1.5 cc. V_2 — 0.5 cc.		++	
25	1 Cl_1 — 1.0 cc.	6/13†	++	
	2 Cl_1 — 1.0 cc. V_2 — 0.5 cc.		++	
26	1 Cl_1 — 0.5 cc.	6/9†	++	
	2 Cl_1 — 0.5 cc. V_2 — 0.5 cc.		++	
27	1 Cl_2 — 1.5 cc.		−	
	2 Cl_2 — 1.5 cc. V_1 — 0.5 cc.	7/27	−	
28	1 Cl_2 — 1.0 cc.		+	
	2 Cl_2 — 1.0 cc. V_1 — 0.5 cc.	6/16†	−	
29	1 Cl_2 — 0.5 cc.		++	
	2 Cl_2 — 0.5 cc. V_1 — 0.5 cc.	7/17†	−	
30	1 Cl_2 — 1.5 cc.		++	
	2 Cl_2 — 1.5 cc. V_2 — 0.5 cc.	6/21	++	
31	1 Cl_2 — 1.0 cc.		−	
	2 Cl_2 — 1.0 cc. V_2 — 0.5 cc.	5/19†	−	
32	1 Cl_2 — 0.5 cc.		−	
	2 Cl_2 — 0.5 cc. V_2 — 0.5 cc.	6/7†	−	

Chick No.	Injection	Date of death or killing	Result 1	Result 2
33	1 Cl_2 — 1.5 cc.		++	
	2 Cl_2 — 1.5 cc. V_3 — 0.5 cc.	6/21†	−	
34	1 Cl_2 — 1.0 cc.		−	
	2 Cl_2 — 1.0 cc. V_3 — 0.5 cc.	6/13†	±	
35	1 Cl_2 — 0.5 cc.		−	
	2 Cl_2 — 0.5 cc. V_3 — 0.5 cc.	8/14	−	
36	1 Cl_3 — 1.5 cc.		−	
	2 Cl_3 — 1.5 cc. V_1 — 0.5 cc.	7/17	−	
37	1 Cl_3 — 1.0 cc.		−	
	2 Cl_3 — 1.0 cc. V_1 — 0.5 cc.	5/28†	−	
38	1 Cl_3 — 0.5 cc.		−	
	2 Cl_3 — 0.5 cc. V_1 — 0.5 cc.	7/17	−	
39	1 Cl_3 — 1.5 cc.		−	
	2 Cl_3 — 1.5 cc. V_2 — 0.5 cc.	8/21	−	
40	1 Cl_3 — 1.0 cc.		−	
	2 Cl_3 — 1.0 cc. V_2 — 0.5 cc.	9/8	−	
41	1 Cl_3 — 0.5 cc.		−	
	2 Cl_3 — 0.5 cc. V_2 — 0.5 cc.	7/4*	−	
42	1 Cl_3 — 1.5 cc.		−	
	2 Cl_3 — 1.5 cc. V_3 — 0.5 cc.	6/2	−	
43	1 Cl_3 — 1.0 cc.		−	
	2 Cl_3 — 1.0 cc. V_3 — 0.5 cc.	9/8	−	
44	1 Cl_3 — 0.5 cc.		−	
	2 Cl_3 — 0.5 cc. V_3 — 0.5 cc.	9/8	−	
45	1 Cl_4 — 1.5 cc.		−	
	2 Cl_4 — 1.5 cc. V_1 — 0.5 cc.	6/9†	−	
46	1 Cl_4 — 1.0 cc.		−	
	2 Cl_4 — 1.0 cc. V_1 — 0.5 cc.	8/14	−	
47	1 Cl_4 — 0.5 cc.		−	
	2 Cl_4 — 0.5 cc. V_1 — 0.5 cc.	7/11†	++	

* No tumors at site of injections. Body cavity full of tumors.

Chick No.	Injection	Date of death or killing	Result 1	Result 2
48	1 Cl_4 — 1.5 cc.			—
	2 Cl_4 — 1.5 cc. V_2 — 0.5 cc.	7/4†		—
49	1 Cl_4 — 1.0 cc.			—
	2 Cl_4 — 1.0 cc. V_2 — 0.5 cc.	7/23		—
50	1 Cl_4 — 0.5 cc.			—
	2 Cl_4 — 0.5 cc. V_3 — 0.5 cc.	7/23		—
51	1 Cl_4 — 1.5 cc.			—
	2 Cl_4 — 1.5 cc. V_3 — 0.5 cc.	6/21†		++
52	1 Cl_4 — 1.0 cc.			—
	2 Cl_4 — 1.0 cc. V_3 — 0.5 cc.	6/7		—
53	1 Cl_4 — 0.5 cc.			—
	2 Cl_4 — 0.5 cc. V_3 — 0.5 cc.	7/23		—

The specific factor Cl_1 was obviously insufficiently treated with chloroform since most of that added to one of the tubes was noted to evaporate from the surface during the first half-hour's incubation. Chicks 47 and 51, injected with the specific factor receiving the largest amount of chloroform, are suggestive. However, No. 47 received V_1, a culture later found to be contaminated, and No. 51 received V_3, an uninoculated chick embryo control. It seems justifiable to look on these as entirely accidental. Certainly one should expect a more striking effect on many of the other chickens before accepting these as evidence of a satisfactory experiment.

Experiment May 28, 1926.—Two lots of 35 cc. each of sand filtrate from desiccated tumor treated with 0.07 cc. and 0.14 cc. respectively, of chloroform, mixed at once with pipette, placed in water bath at 37°, and mixed again at the end of half an hour; after a total of 3 hours, chloroform removed *in vacuo;* noted as Cl_1 and Cl_2 respectively.

The following virus preparations used, all 4 day anaerobics:

V_1—Rat Sarcoma 10—sterile on culture.

V_2—Mouse Sarcoma 180—sterile on culture.

V_3—Mouse Carcinoma D Br B—sterile on culture.

V_4—Rat embryo.

V_5—Rat placenta.

Chick No.	Injection	Date of death or killing	Result 1	Result 2
54	1 Cl_1 — 1.5 cc.			
	2 Cl_1 — 1.5 cc. + V_1 — 0.5 cc.	Missing		
55	1 Cl_1 — 1.0 cc.		++	
	2 Cl_1 — 1.0 cc. + V_1 — 0.5 cc.	7/6	−*	
56	1 Cl_1 — 0.5 cc.		−	
	2 Cl_1 — 0.5 cc. + V_1 — 0.5 cc.	9/8**	−	
57	1 Cl_1 — 1.5 cc.		++	
	2 Cl_1 — 1.5 cc. + V_2 — 0.5 cc.	6/27†	−	
58	1 Cl_1 — 1.0 cc.			
	2 Cl_1 — 1.0 cc. + V_2 — 0.5 cc.	Missing		
59	1 Cl_1 — 0.5 cc.		−	
	2 Cl_1 — 0.5 cc. + V_2 — 0.5 cc.	6/16†	−	
60	1 Cl_1 — 1.5 cc.		−	
	2 Cl_1 — 1.5 cc. + V_3 — 0.5 cc.	8/6	−	
61	1 Cl_1 — 1.0 cc.		−	
	2 Cl_1 — 1.0 cc. + V_3 — 0.5 cc.	7/4	++	
62	1 Cl_1 — 0.5 cc.		−	
	2 Cl_1 — 0.5 cc. + V_3 — 0.5 cc.	6/29	−	
63	1 Cl_1 — 1.5 cc.		−	
	2 Cl_1 — 1.5 cc. + V_4 — 0.5 cc.	6/1†	−	
64	1 Cl_1 — 1.0 cc.		−	
	2 Cl_1 — 1.0 cc. + V_4 — 0.5 cc.	6/25†	++	
65	1 Cl_1 — 0.5 cc.		++	
	2 Cl_1 — 0.5 cc. + V_4 — 0.5 cc.	7/24†	−	
66	1 Cl_1 — 1.5 cc.		−	
	2 Cl_1 — 1.5 cc. + V_5 — 0.5 cc.	6/10†	−	
67	1 Cl_1 — 1.0 cc.		−	
	2 Cl_1 — 1.0 cc. + V_5 — 0.5 cc.	7/26†	++	
68	1 Cl_1 — 0.5 cc.		−	
	2 Cl_1 — 0.5 cc. + V_5 — 0.5 cc.	9/8**	±	

* A tumor formed at this site in 3 weeks but had completely retrogressed when the animal was killed.

** Still living.

Chicks 69 to 83 were injected according to the same scheme, with Cl_2 instead of Cl_1. No tumors whatever resulted in these animals.

A large per cent of these chicks died of a coccidial infection too soon to show tumors. Most of the others were in bad condition and a number have died in the course of the summer in an extremely emaciated condition. It is sufficient to point out here again the lack of uniformity of results.

Experiment June 18, 1925.—Desiccated tumor. Cl_1 and Cl_2 contained respectively 0.05 cc. and 0.10 cc. chloroform to 25 cc. filtrate.

V_1—Mouse Carcinoma D Br B culture.

V_2—Primary aerobic Rous sarcoma culture, 8 days old.

V_3—Mouse embryo plus mouse placenta.

V_4—Chick embryo.

V_1, V_3, and V_4 were incubated 4 days in anaerobic jar.

All preparations were sterile on culture.

Chick No.	Injection	Date of death or killing	Result
84	1 Cl_1 — 1.0 cc.		—
	2 Cl_1 — 0.5 cc. + V_1 — 0.5 cc.	8/16	—
85	1 Cl_1 — 1.0 cc.	7/16	++
	2 Cl_1 — 0.5 cc. + V_1 — 0.5 cc.		++
86	1 Cl_1 — 1.0 cc.		++
	2 Cl_1 — 0.5 cc. + V_1 — 0.5 cc.	8/10†	++
87	1 Cl_1 — 1.0 cc.		++
	2 Cl_1 — 0.5 cc. + V_3 — 0.5 cc.	7/23†	—
88	1 Cl_1 — 1.0 cc.		++
	2 Cl_1 — 0.5 cc. + V_3 — 0.5 cc.	7/23†	—
89	1 Cl_1 — 1.0 cc.		—
	2 Cl_1 — 0.5 cc. + V_3 — 0.5 cc.	7/13†	++
90	1 Cl_1 — 1.0 cc.		—
	2 Cl_1 — 0.5 cc. + V_3 — 0.5 cc.	8/7†	++
91	1 Cl_1 — 1.0 cc.		—
	2 Cl_1 — 0.5 cc. + V_3 — 0.5 cc.	7/1†	—
92	1 Cl_1 — 1.0 cc.		—
	2 Cl_1 — 0.5 cc. + V_3 — 0.5 cc.	9/8*	—
93	1 Cl_1 — 1.0 cc.		—
	2 Cl_1 — 0.5 cc. + V_4 — 0.5 cc.	6/29†	—
94	1 Cl_1 — 1.0 cc.		—
	2 Cl_1 — 0.5 cc. + V_4 — 0.5 cc.	8/16	—
95	1 Cl_1 — 1.0 cc.		—
	2 Cl_1 — 0.5 cc. + V_4 — 0.5 cc.	8/27	—

Nos. 96 to 108 done exactly as the above, with Cl_2 instead of Cl_1, were all negative on both sides.

* Still living.

One need only refer to the first seven chickens of this protocol to note the type of variations which occur in these experiments.

Experiment June 27.—Sand filtrate from a fresh tumor was used in this experiment. Four 15 cc. lots, designated Cl_1, Cl_2, Cl_3, Cl_4, were treated as usual with 0.045, 0.09, 0.18, and 0.36 cc. chloroform respectively.

V_1—Jensen rat sarcoma.

V_2—Uninoculated chick embryo.

Both grown 4 days anaerobically and sterile on subculture to blood agar.

Chick No.	Injection	Date of death or killing	Results
109	1 Cl_1 — 1.0 cc.		−
	2 Cl_1 — 0.5 cc. + V_1 — 0.5 cc.	7/8†	−
110	1 Cl_1 — 1.0 cc.		++
	2 Cl_1 — 0.5 cc. + V_1 — 0.5 cc.	7/10†	++
111	1 Cl_1 — 1.0 cc.		++
	2 Cl_1 — 0.5 cc. + V_1 — 0.5 cc.	7/13†	++
112	1 Cl_1 — 1.0 cc.		−
	2 Cl_1 — 0.5 cc. + V_2 — 0.5 cc.	7/13†	−
113	1 Cl_1 — 1.0 cc.		++
	2 Cl_1 — 0.5 cc. + V_2 — 0.5 cc.	7/13†	−
114	1 Cl_1 — 1.0 cc.		++
	2 Cl_1 — 0.5 cc. + V_2 — 0.5 cc.	7/10†	−
115	1 Cl_2 — 1.0 cc.		++
	2 Cl_2 — 0.5 cc. + V_1 — 0.5 cc.	7/16†	++
116	1 Cl_2 — 1.0 cc.		++
	2 Cl_2 — 0.5 cc. + V_1 — 0.5 cc.	7/16†	++
117	1 Cl_2 — 1.0 cc.		++
	2 Cl_2 — 0.5 cc. + V_1 — 0.5 cc.	7/19	++
118	1 Cl_2 — 1.0 cc.		++
	2 Cl_2 — 0.5 cc. + V_2 — 0.5 cc.	7/15†	++
119	1 Cl_2 — 1.0 cc.		−
	2 Cl_2 — 0.5 cc. + V_2 — 0.5 cc.	7/10†	−
120	1 Cl_2 — 1.0 cc.		++
	2 Cl_2 — 0.5 cc. + V_2 — 0.5 cc.	7/13†	++
121	1 Cl_3 — 1.0 cc.		−
	2 Cl_3 — 0.5 cc. + V_1 — 0.5 cc.	7/4†	−

Chick No.	Injection	Date of death or killing	Results
122	1 Cl_3 — 1.0 cc.		+
	2 Cl_3 — 0.5 cc. + V_1 — 0.5 cc.	8/15†	−
123	1 Cl_3 — 1.0 cc.		+
	2 Cl_3 — 0.5 cc. + V_1 — 0.5 cc.	7/17†	−
124	1 Cl_3 — 1.0 cc.		++
	2 Cl_3 — 0.5 cc. + V_2 — 0.5 cc.	7/31†	++
125	1 Cl_3 — 1.0 cc.		++
	2 Cl_3 — 0.5 cc. + V_2 — 0.5 cc.	7/27†	−
126	1 Cl_3 — 1.0 cc.		−*
	2 Cl_3 — 0.5 cc. + V_2 — 0.5 cc.	8/10†	−
127	1 Cl_4 — 1.0 cc.		−
	2 Cl_4 — 0.5 cc. + V_1 — 0.5 cc.	8/26	++
128	1 Cl_4 — 1.0 cc.		−
	2 Cl_4 — 0.5 cc. + V_1 — 0.5 cc.	7/1	−
129	1 Cl_4 — 1.0 cc.		−
	2 Cl_4 — 0.5 cc. + V_1 — 0.5 cc.	9/8**	−
130	1 Cl_4 — 1.0 cc.		+
	2 Cl_4 — 0.5 cc. + V_2 — 0.5 cc.	8/16	−
131	1 Cl_4 — 1.0 cc.		−
	2 Cl_4 — 0.5 cc. + V_2 — 0.5 cc.	8/7	−
132	1 Cl_4 — 1.0 cc.		++
	2 Cl_4 — 0.5 cc. + V_2 — 0.5 cc.	8/3	−

* This chicken had previously shown a small tumor at Site 1, which at time of death (extreme emaciation), had retrogressed.

** Still living.

In this experiment it is evident that in Cl_4, a preparation was used which just failed to infect most of the chickens. Active agent was obviously still present, yet there was no evidence of activation by anything present in the virus preparations.

Experiment July 18, 1926.—Sand filtrate from a fresh tumor made as usual. Cl_1, Cl_2, and Cl_3 contained respectively 0.09, 0.18, and 0.36 cc. chloroform to 15 cc. filtrate.

V_1—Jensen sarcoma primary culture.

V_2—Mouse embryo and placenta.

Both incubated 4 days anaerobically, sterile by subculture.

Chick No.	Injection	Date of death or killing	Results
133	1 Cl_1 — 1.0 cc.		−
	2 Cl_1 — 0.5 cc. + V_1 — 0.5 cc.	9/8*	−
134	1 Cl_1 — 1.0 cc.		++
	2 Cl_1 — 0.5 cc. + V_1 — 0.5 cc.	9/10	±
135	1 Cl_1 — 1.0 cc.		−
	2 Cl_1 — 0.5 cc. + V_1 — 0.5 cc.	9/8*	−
136	1 Cl_1 — 1.0 cc.		+
	2 Cl_1 — 0.5 cc. + V_2 — 0.5 cc.	8/21†	−
137	1 Cl_1 — 1.0 cc.		++
	2 Cl_1 — 0.5 cc. + V_2 — 0.5 cc.	8/29†	−
138	1 Cl_1 — 1.0 cc.		−
	2 Cl_1 — 0.5 cc. + V_2 — 0.5 cc.	8/16	−
139	1 Cl_2 — 1.0 cc.		−
	2 Cl_2 — 0.5 cc. + V_1 — 0.5 cc.	9/8*	±
140	1 Cl_2 — 1.0 cc.		−
	2 Cl_2 — 0.5 cc. + V_1 — 0.5 cc.	9/8*	−
141	1 Cl_2 — 1.0 cc.		−
	2 Cl_2 — 0.5 cc. + V_1 — 0.5 cc.	9/8*	−
142	1 Cl_2 — 1.0 cc.		−
	2 Cl_2 — 0.5 cc. + V_2 — 0.5 cc.	9/8*	±
143	1 Cl_2 — 1.0 cc.		−
	2 Cl_2 — 0.5 cc. + V_2 — 0.5 cc.	9/8*	−
144	1 Cl_2 — 1.0 cc.		−
	2 Cl_2 — 0.5 cc. + V_2 — 0.5 cc.	9/8*	−

* Still living.

Chick No.	Injection	Date of death or killing	Results
145	1 Cl_3 — 1.0 cc.		—
	2 Cl_3 — 0.5 cc. + V_1 — 0.5 cc.	9/8*	—
146	1 Cl_3 — 1.0 cc.		+
	2 Cl_3 — 0.5 cc. + V_1 — 0.5 cc.	9/8*	—
147	1 Cl_3 — 1.0 cc.		—
	2 Cl_3 — 0.5 cc. + V_1 — 0.5 cc.	9/8	—
148	1 Cl_3 — 1.0 cc.		—
	2 Cl_3 — 0.5 cc. + V_2 — 0.5 cc.	9/8*	—
149	1 Cl_3 — 1.0 cc.		—
	2 Cl_3 — 0.5 cc. + V_2 — 0.5 cc.	9/8*	—
150	1 Cl_3 — 1.0 cc.		++
	2 Cl_3 — 0.5 cc. + V_2 — 0.5 cc.	8/10	—

Here again there is no evidence of reactivation of chloroform filtrates still containing slightly active agent, by the virus preparations used.

GENERAL DISCUSSION.

If one summarizes the results of the larger experiments, and enumerates the results only of those chickens into which chloroform filtrates were injected, giving less than about 50 per cent control takes, the following result is obtained. 70 chickens injected with specific factor in one side, and with a mixture of specific factor and virus into the opposite side were completely negative. Four were positive on both sides. Fifteen showed a take from the specific factor alone, and twelve from the mixture only. These occasional single takes with control or mixture were interspersed in the same experiments, and the figures would seem to establish definitely that such results in work of this kind are completely without significance. We should like, therefore, to suggest that in the presentation of further studies on this subject, enough consecutive protocols be published to make very clear to the reader the general trend of the experiments, and that to guard even here against accidental variations, plenty of controls be included in each experiment.

If we assume, however, as we must, that all the work done so far

has been based upon amply controlled experiments, is there any way in which the discordant results can be either harmonized or explained? There are four types of findings to be considered. Chloroform filtrate from the Rous sarcoma has been found to be reactivated (1) by nothing (Harkins, Schamberg, Kolmer, and Kast, and the writer); (2) only by virus obtained from tumors (Gye); (3) by extracts of tumor, embryonic tissue, and placenta, *i.e.*, rapidly growing cells (Murphy and Cori); and (4) by extracts of tumors, embryo tissue, and normal liver and kidney (Flu).

In Gye's original paper he does not mention controls of normal tissue. In a later discussion of his work (7), however, he stated that he had controls with media containing embryonic tissue with negative results and subsequent information satisfies us that Dr. Gye has given the attention to this form of control which one would expect from such an experienced worker. Murphy, in his turn, does not mention attempts to use normal adult tissues, such as liver and kidney. One is therefore left in doubt as to whether his results might not harmonize with those of Flu. Obviously a positive experiment of this type must have more weight than a negative, and one cannot help feeling that a sufficiently controlled series of experiments in the hands of those who appear to be able to make the phenomenon work at all, should settle this point. Should the activating principle obtained by Gye from tumor tissue be obtainable also from normal tissues, obviously his hypothesis must be incorrect.

Why should an experiment which is simple in theory, and not highly complicated in practical aspects, lead to three or four different results in as many laboratories? The variable factors entering into the work are three, or at most four, *i.e.*, the tumor itself, the specific factor, the virus, and possibly the strain of chickens used. The last of these can probably be ruled out, since there is pretty general agreement about the susceptibility of Barred Plymouth Rock chickens.

The tumor, however, is a completely unknown element. It is a fact, well recognized by those who have had experience with transplantable mammalian tumors, that a most extreme variation, almost periodic in some cases, is shown by neoplasms in the percentage of takes in susceptible animals. For this, no adequate explanation has been advanced. Moreover, it has been shown by Gye (6) that the

Rous sarcoma may suddenly change its properties and for several generations be not transmissible by filtrates, glycerolated or desiccated tissue, but only with living cells, and then as suddenly regain its usual properties. It is conceivable, therefore, that in many generations of growth in different laboratories, tumors originating from a common source should have so altered their properties along somewhat different lines as to account for marked variability in behaviour in such a method as Gye's. This could be completely ruled out only by an exchange of tumor strains and might be worth checking up.

The specific factor can be *proved* to be satisfactory only if it is *known* that the virus is right, and *vice versa*. In other words, in the case of negative results such as our own, it is almost impossible to say which factor is at fault, or whether both are wrong. For example, does our chloroform, especially purified though it has been, contain a trace of something which destroys the chemical specific factor more rapidly than the virus? If not, then in most of our larger experiments we have had preparations which should have been satisfactory, for a range of destructive action has been shown from obviously too little to a probable excess, through the range between the two, some of which should have represented the correct amount. No such indirect method of control is possible, however, for the viruses. One can only hope to get satisfactory cultures by following as closely as possible the directions set down by Gye. We do not consider here the possibility of checking them by ultra-violet photography according to the method of Barnard (8), because of obvious technical difficulties. Very slight differences in culture media, contamination with other organisms perhaps not recognizable by the usual methods of smear and subculture, or some entirely unrecognized difference in method may have meant that we have never once had a suitable virus preparation. It is worth noting, in this connection, that our cultures were often slightly turbid, although no bacteria could be demonstrated in them. We believe the turbidity to have been due to the acid reaction known to be developed by tumor tissue in the presence of certain sugars. If this takes place in a solution containing tissue juices and serum, one expects a certain amount of clouding or precipitation of protein, and we have attributed the phenomenon, perhaps wrongly, to this cause.

If it should subsequently be shown that Gye is right, we should be

inclined to consider that our virus preparations have been consistently at fault, since error in regard to the other factors seems less likely.

The impossibility of controlling any of the three factors entering into the work of course detracts considerably from the value of purely negative results like our own. But we feel that in a subject as important as this, any evidence bearing upon the problem should be placed as soon as possible at the disposal of others who may be interested.

CONCLUSIONS.

It is obviously impossible to draw definite conclusions as to the significance of the differences between our work and Gye's, and still less, of the differences between Gye's work and that of Murphy and of Flu. We can only say that in a fairly large series of experiments, extending over a period of 12 months, we have had absolutely no indication of the necessity of two factors in the production of the Rous sarcoma. In other words, we have been unable to duplicate either the results of Gye or the modified confirmations of his work by Murphy and Flu. We have shown that uncontrollable local and individual variations may produce results in occasional chicks which simulate satisfactory experiments, but when viewed as a whole, mean nothing. Because of the conflicting nature of results obtained by those who have undertaken to repeat the work, and on account of the difficulty of controlling all factors involved, we do not feel that it may be stated definitely that Gye's theory of the cause of cancer is wrong. On the other hand the theory apparently needs more evidence in its support if it is to receive further serious consideration. It is suggested, in order to untangle the subject as rapidly as possible, that future publications should include sufficient consecutive protocols to make the trend of the experiments obvious to the reader.

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DIET AND TISSUE GROWTH.

IV. The Rate of Compensatory Renal Enlargement after Unilateral Nephrectomy in the White Rat.*

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The enlargement of the surviving kidney after unilateral nephrectomy is a well known and generally accepted fact. On the other hand, the character of this enlargement has been long disputed and has been the subject of many investigations. The literature on this point has been summarized by Arataki (1926, *b*) who, in his own studies on rats, found an increase in the size of both the glomeruli and the tubular systems, an hypertrophy, together with an actual cellular increase of the supporting tissue, an hyperplasia. He also believes that there is an hyperplasia of the constituent cells of the glomeruli and tubules.

On equally important phases of this subject, namely the rate of growth and the dietary factors concerned, there is a striking paucity of data. It is the purpose of this communication to report experimental studies on these questions.

As a result of the metabolic processes the nutritive fluid surrounding the tissues is vastly different both physically and chemically from the food ingested. The composition of this fluid medium of the cells changes comparatively little from day to day in a given species. It has been found possible experimentally to vary the quality of the food ingested within surprisingly wide limits without interfering with the normal functions of the organism. On the other hand, investigations on the effects of extreme or abnormal rations

* A preliminary report of a portion of this work was presented in the *Proceedings of the Society for Experimental Biology and Medicine* (Moise, T. S., and Smith, A. H., *Proc. Soc. Exp. Biol. and Med.*, 1925–26, xxiii, 561).

have disclosed the fact that there are certain unique dietary demands made by the body if growth and optimal functional activity are to be maintained. Insufficient energy intake, lack of essential amino acids, deficiency in certain inorganic salts and absence of accessory food factors all result in the disturbance of activity of certain organs and, grossly, in inhibition of growth. Recent studies have indicated, however, that when stunting occurs certain of the organ systems show a definite persistence of growth (Winters, Smith and Mendel). Smith and Moise (1924) have shown that injured liver tissue in rats is repaired on diets which are inadequate for growth of the body as a whole. It appears that certain organs are more sensitive to dietary insufficiency than others. The relation of diet to tissue growth is thus an intricate one and its investigation merits extension to other organs and systems.

Nothnagel (1886) early came to the conclusion that compensatory renal enlargement is a function of the composition of the blood. That this phenomenon is caused by some stimulus to function was later shown by Sacerdotti (1896) who, after performing unilateral nephrectomy, observed no increase in size of the remaining kidney when the animal fasted. However, when blood from a dog with total nephrectomy was injected into a normal animal without increasing the blood volume, there was evidence of renal enlargement. Enlarged kidneys have been observed in young rats grown on high protein foods and in mature rats given such diets, when both kidneys were present (Osborne, Mendel, Park and Darrow (1923), Reader and Drummond (1925), Jackson and Riggs (1926)). It appears from these experiments that in intact animals the level of non-protein nitrogen of the blood conditioned either by retention, on the one hand, or by abnormally high level of protein in the ration, on the other, influences directly the enlargement of the kidneys. That the same factor plays a large part in causing compensatory enlargement is shown in the present study.

The rate at which the total amount of renal tissue increases in response to these rather specific changes in blood composition is not only of academic interest but also of practical value. Osborne, Mendel, Park and Winternitz (1925) reported enlargement of the two kidneys in rats after they had been given protein-rich food for 1 week. It is of considerable importance in renal surgery to obtain similar statistics concerning the remaining kidney after nephrectomy. So far as we know such information on experimental animals is not available in the literature. Hinman (1923) gives data for the compensatory "hypertrophy" of the right kidney in rats after ligation of the left ureter without removing the kidney. In view of the scarcity of the published data, it seemed desirable to institute an extensive series of experiments on the problem of compensatory renal enlargement after unilateral nephrectomy.

It is obvious that any practical value of such results does not lie in the importance of the size of the organ *per se* but only in its function as correlated with its size. It has been pointed out by Oliver (1924) that in nephrectomized rabbits the weight of the kidneys is not a true index of the functional activity of the organ because the convoluted tubules and the glomeruli grow at unequal rates and urea is largely excreted in the tubules (Oliver, 1921). Studies of renal function to supplement the present investigation are in progress.

EXPERIMENTAL PART.

Methods.—The plan of the experiment was to remove the right kidney, place the animal immediately on the ration to be used and after a definite time interval to remove the remaining kidney for study. This procedure was carried out with the "standard" food, a diet providing the minimal amount of protein for optimal growth and maintenance and with the "high protein" food, which was unusual in that it contained a high concentration of protein. A third phase involved the study of the degree of compensatory renal enlargement after a constant time but with the animals eating diets containing increasing amounts of protein.

Adult white rats were used to eliminate the growth factor in renal enlargement, since it is well known that the organs increase in size as the body grows. Although a fluctuation in body weight occurred during the periods following nephrectomy, the authors believe that the change in size of the kidneys here recorded is one of practically uncomplicated compensation for the loss of kidney tissue as the result of the initial nephrectomy with the protein content of the diet as the only variable factor. An effort was made to use only male rats but in the early parts of the study non-pregnant females also were employed. None of the animals used had been given other than "stock" diets prior to being used in the present work. Individual variation, the chief difficulty inherent in animal experimentation, must be borne in mind in interpreting the present data. This variation may arise from differences either in heredity or in previous environment, which, in turn, might affect not only the gross reaction of the animal to the diet (efficiency of digestion and absorption) but also the response of the kidney cells to changes in blood composition. An effort was made therefore to use a relatively large number of rats for each experimental test period in order to eliminate as far as possible the uncertain factor of individual variation. It was planned to have twelve animals for each period in the study. In a few cases, accidental death reduced the number while in most instances more than twelve survived. In all, the data on considerably more than 300 rats were used in the final calculations.

In all the experiments diets composed of mixtures of purified foodstuffs were used. Casein furnished the protein, lard provided the fat, raw corn starch was used as a source of carbohydrate and an artificial salt mixture supplied the inorganic requirements. Cod liver oil mixed with the other ingredients and dried yeast fed apart from the food were used as sources of vitamines. In all the experiments the food and fresh tap water were given in unlimited quantity. It is im-

portant to insure an abundant supply of water, for with the protein-rich diets the large quantities of urea resulting from the metabolism of the protein constitute a potent diuretic and the increased volume of urine necessitates augmented fluid intake. In Table I is given the percentage composition of the various diets employed together with the approximate portion of the energy provided by the constituent foodstuffs. In planning the rations the aim has been to vary the proportion of protein while the other essential parts of the diet, *i.e.*, the salts and vitamines, remained unchanged. The caloric value of the diets containing 60 per cent or less of casein is approximately 5.3 Calories per gm. food. As the casein

TABLE I.

Composition of Experimental Diets.

	"Standard" food		Diet 30		Diet 45		Diet 60		Diet 75		"High protein" food		Diet 90	
	Part of diet	Part of total calories												
	per cent	*per cent*												
Protein (casein)[1]	18	14	30	23	45	35	60	46	75	60	85	76	90	85
Carbohydrate (raw cornstarch)	51	39	39	30	24	18	9	7						
Fat (lard)	22	47	22	47	22	47	22	47	17	40	7	24		
Fat (cod liver oil)	5		5		5		5		5		4		7	15
Salt mixture[2]	4		4				4		3		4		3	

700 ~~300~~ mg. dried yeast daily given with each of the diets.

[1] A commercial product containing 13 per cent nitrogen.

[2] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

is increased above 60 per cent the energy value per gm. food becomes progressively less.

The method of caging used is the same as that heretofore described (Smith, Cowgill and Croll, 1925).

The technique of the operation was as follows: The rat was anesthetized with ether, the hair on the right flank was clipped closely and the area was painted with tincture of iodine. Under aseptic precautions an incision was made just below and parallel to the right costal margin. The peritoneum was incised and the kidney was lifted up by passing a small aneurysm needle through the pelvis. The perirenal fat was quickly dissected away, the pedicle ligated *en masse* and the kidney removed.

CORRECTIONS.

In Vol. xlv, No. 2, page 266, last line of Table I, for "300 mg. dried yeast daily given with each of the diets" read "700 mg. dried yeast daily given with each of the diets."

The wound was sutured in two layers and a thin collodion dressing applied. In the beginning of the experiments there was a small operative mortality from hemorrhage but this was eliminated by more complete dissection of the pedicle and thereafter there were no operative deaths. Wound infection was a negligible factor.

Early in the study the weight of the kidneys was obtained as well as linear measurements of the length, width and thickness. This method was finally discarded as unreliable on account of variations in the measurements of the small soft vascular viscus with slight pressure or with a change in the position of the organ to be measured. Immediately following its removal, the kidney, free of fat and hilic structures, was weighed in a stoppered bottle on an analytical balance. The manipulation was so standardized that the differences in amount of blood lost at various times is believed to have been negligible. After weighing, some of the kidneys were prepared for histological study and others were used for determinations of total solids.

The degree of enlargement was calculated as follows: On account of the change in the body weight of the rats during the period of study, the original (right) kidney weight was compared to that of one of Donaldson's (1924) rats of the same body weight and a factor obtained. The weight (A) of the enlarged kidney of the experimental rat was then multiplied by this factor to enable a direct comparison with Donaldson's figure to be made. From this product was subtracted the weight (B) of one kidney of a Donaldson rat weighing the same as the experimental rat at the end of the period. The difference is the actual enlargement and this divided by the weight (B) gives the enlargement in percentage. In view of the loss in body weight immediately following nephrectomy this method of calculation probably magnifies the degree of enlargement in the early periods, especially on the high protein diets. An example of the method of calculation is given below.[1]

Many measurements of both kidneys in normal rats have shown that they are either of the same weight or that the right is slightly heavier than the left. These observations are confirmed by Arataki (1926, *a*) who states that "on the average between birth and 350 days

	Initial body weight	Final body weight	Original kidney weight	Final kidney weight
	gm.	*gm.*	*gm.*	*gm.*
[1] Average of 12 rats present experiment	287	277	1.126	2.419
Donaldson's corresponding values	286.5	277.1	1.175	1.140

$$\text{Correction factor} = \frac{1.175}{1.126} = 1.04$$

$$\text{Corrected final kidney weight} = 2.419 \times 1.04 = 2.515 \text{ gm.}$$

$$\text{Difference (enlargement)} = 2.515 - 1.140 = 1.375 \text{ gm.}$$

$$\text{Degree of enlargement} = \frac{1.375}{1.140} \times 100 = 121 \text{ per cent}$$

the right kidney is 2.1 per cent heavier than the left in the male and 2.3 per cent heavier in the female. These differences are slight, but since the right kidney is also more often the heavier, they would appear to represent a real, though small, inequality." This fact introduces an error in some of the experiments in that the calculated percentages of enlargement are too low. However, these differences are so slight in view of the inevitable experimental difficulties involved in such work that they may be disregarded.

TABLE II.

Degree of Gross Enlargement of Remaining Kidneys of Rats Given "Standard" Food.

Interval after nephrectomy	Number of animals	Mean degree of enlargement
days		*per cent*
3	11	5
9	10	14
14	15	16
21	12	24
28	15	17
42	12	28
56	15	23
90	11	35
120	13	44
150	11	48

Results.

Changes in Body Weight.—After nephrectomy there was invariably a loss in weight of the experimental animals. The weight remained below the preoperative level in the group on "standard" food for a period of 2 to 4 weeks, by which time it had returned to the previous value and in many cases increased somewhat as the experiment progressed.

In the group on the "high protein" food the body weight decreased within 3 to 4 weeks following nephrectomy to a new level, often 30 or 40 gm. below the preoperative value, at which it remained throughout the experiment or only slowly rose after a period of 4 or 5 months toward the preoperative level. Concomitant with the initial loss in weight there was observed a decreased consumption of food. As

the body weight of this group of rats became stabilized at this new level, the food intake rose until these rats were eating more gm. of food than those on the "standard" diet but, due to the lower caloric value of the "high protein" food, were actually ingesting less energy (see Smith and Carey, 1923). It appears, therefore, that after the immediate shock of the operation, the rise in nitrogenous waste products in the blood acts as a systemic depressant which is

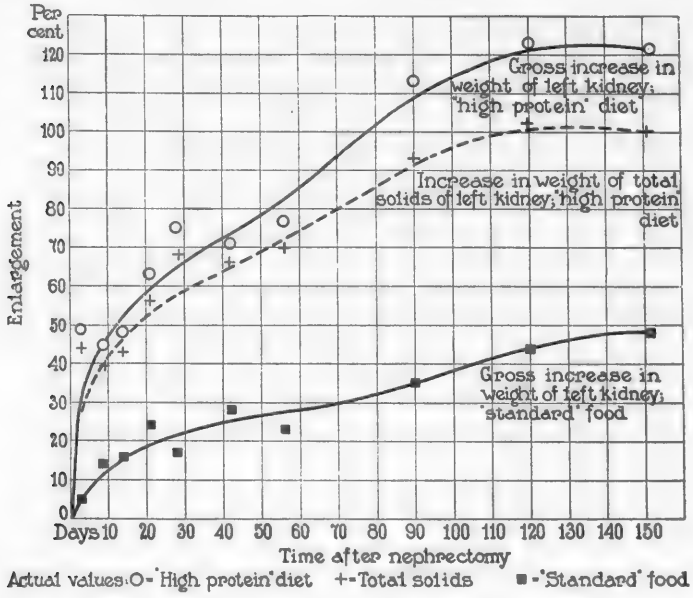

CHART 1. Rate of compensatory enlargement of remaining kidney after unilateral nephrectomy.

reflected in the appetite and body weight of the animal. These observations indicate that in mature rats, physiological well being may be adversely influenced not only by the well known method of decreasing the food intake but also by interfering with the excretion of substances which are primarily waste products. It is more than likely that a definite demonstration of this thesis could be produced in growing rats on protein-rich dietaries.

Rate of Kidney Enlargement.—The results of the study on the rats fed the "standard" food are summarized in Table II and in Chart 1. The individual values for the 3 day period were exceedingly variable, in some of the cases a decrease in size being observed. The recorded value of 5 per cent enlargement after 3 days is therefore only approximately correct. As may be seen from the data there is a rapid increase in the compensatory enlargement of the remaining kidney within the first 3 weeks following unilateral nephrectomy. The left kidney is about one-fifth heavier than the control at this time. The increase after 21 days and until the 120 day period appears to be progressive but at a definitely slower rate (approximately 3 per cent

TABLE III.

Degree of Gross Compensatory Enlargement after 21 Days on Diets of Varying Protein Content.

Casein in diet	Number of animals	Mean degree of enlargement
per cent		*per cent*
18	12	24
30	13	37
45	13	31
60	13	43
75	13	56
85	15	63
90	7	77

in 10 days). The data show that no significant enlargement takes place between the 120th and the 150th days. Hinman (1923) reported that in rats with one ureter ligated, the remaining kidney had reached its maximum compensatory enlargement on the average after 20 to 30 days which does not agree with what is here reported for nephrectomized rats. On the other hand, the data of Addis, Myers and Oliver (1924), when calculated in a way similar to the present method, show that at 106 to 125 days after nephrectomy the remaining kidney of the rabbit shows approximately 32 per cent enlargement, somewhat less than the value indicated for rats in the present study. The details of the diets in either of the above papers are not mentioned.

That the sudden response observed in the early periods is a reaction to the blood composition and the consequent increased demand for renal activity seems probable. Although Bürger and Grauhan (1923) have shown that after nephrectomy in the human there is a pronounced increase in nitrogen excretion, the very important part played by the nature of the diet is shown subsequently in the present study.

The second phase of the experiment demonstrates that dietary protein is at least one of the factors conditioning the compensatory enlargement of the kidney. The plan was altered somewhat for these tests. It was observed in the preceding section that on the

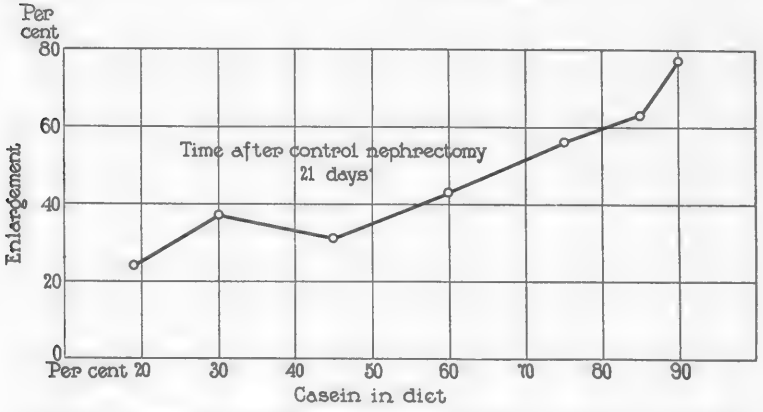

CHART 2. Relation of renal compensatory enlargement to the amount of dietary protein.

"standard" food a marked degree of enlargement could be demonstrated in 21 days. In this second phase the time interval after the first nephrectomy was maintained constant at 21 days and diets with increasing concentrations of protein were used with different groups of rats. Table I shows the rations used.

In Table III are given the data for Chart 2 which shows the degree of enlargement plotted against the per cent of casein in the food. No attempt has been made to draw a "smoothed" curve but, as shown, it is plain that there is almost a direct proportion between the degree of compensatory enlargement and the concentration of dietary protein. In these experiments the catabolic non-

protein nitrogen of Bürger and Grauhan (1923) is constant, since the animals were all treated in the same way and the measurements were made at the same time. Moreover, their published protocols show that in humans the catabolic nitrogen is eliminated within a week. It is obvious, therefore, that the enlargement illustrated by the present data is brought about by some condition depending primarily on the composition of the diet, more specifically, the protein concentration.

What is the maximum compensatory enlargement to which the remaining kidney may be forced under stress resulting from excess dietary protein? To answer this question a series of experiments was planned identical with the first series except that the "high protein" food was used (see Table I). This ration, though extreme, contained all the recognized dietary essentials for growth and maintenance. The data obtained are summarized in Table IV and expressed graphically in Chart 1. The curve showing the rate of enlargement is similar in shape to that of the first series with "standard" food except that the values are much greater. There is the analogous rapid increase in size within the early intervals followed by a slower increase to a maximum. However, where, in the first group on "standard" food, the enlargement at 30 days was about 20 per cent, on the "high protein" diet in a similar length of time the remaining kidney weighed 70 per cent more than the control. The increase progressed at the rate of about 7 per cent in 10 days to the 90th day when the compensatory enlargement was about 113 per cent. From the 90th to the 120th day the rise is much slower and from the 120th day to the 150th day there appears to be no significant increase, the value at 150 days being 121 per cent. It seems probable from these data that even under the stimulus of such a protein-rich diet as here used the maximum enlargement is reached little sooner than on the "standard" food with a relatively low protein content. These combined data show the tremendous physiological reserve possessed by the rat kidney and provide striking evidence for Meltzer's (1906) "factor of safety" in the organism.

The results suggest several questions. Does the increased kidney weight observed after compensatory enlargement result from a *bona fide* increase in renal tissue or is it due to an accumulation of fluid in

the kidney? Many comparisons have been made between the total solids of normal kidneys and of those showing the extreme enlargement on the protein-rich diet. Table V summarizes the data bearing on this point. The average percentage of total solids in the enlarged kidneys in the longer periods, *i.e.*, from 90 to 150 days, is lower than that of normal kidney tissue and the difference is a real one. The total solids per cent of the enlarged kidneys of the 21 to 56 day group is larger but there still is a small though significant variation from the normal. The values for the 3 to 14 day group approaches that of the normal slightly more closely than those of the other two

TABLE IV.

Degree of Compensatory Enlargement of Kidneys of Rats Given "High Protein" Food.

Interval after nephrectomy	Number of animals	Mean degree of enlargement	Mean increase of total solids
days		*per cent*	*per cent*
3	12	49	44
9	13	45	40
14	13	48	43
21	15	63	56
28	14	75	68
42	15	71	64
56	14	77	70
90	10	113	93
120	12	123	102
150	12	121	100

groups. With these data the net tissue enlargement has been calculated for the group on "high protein" food and the results are shown in Chart 1. The degrees of compensatory renal enlargement discussed heretofore in this paper are the gross increase in size, *i.e.*, including the water, the basis upon which other reports in the literature have been made. It is thus obvious that the renal enlargement resulting from extreme conditions of stimulation to functional activity, while a real one, is accompanied by a definite increase in the water content of the kidney and that this is proportional to the time interval during which the animal is given the experimental diet.

Arataki (1926, *b*) has shown that the compensatory enlargement of the remaining kidney is due mainly to the hypertrophy of the glomeruli and tubules. It is possible that the increase in the fluid content of the enlarged kidneys observed in the present experiments is due to its being mechanically retained in the enlarged tubules.

TABLE V.

Total Solids of Kidneys.

Group	Number of rats in groups taken	Degree of enlargement	Mean total solids	Probable error	Significant difference from normal* $\frac{D}{PEd}$
		per cent	*per cent*	*per cent*	
Normal	25		22.3 ± 0.78	± 0.101	
*"High protein" food***					
90, 120 and 150 day periods	15	113–123	20.2 ± 0.45	± 0.078	16.1 Positive
21, 28, 42 and 56 day periods	19	64–77	21.4 ± 0.92	± 0.15	5.0 Positive
3, 9 and 14 day periods	14	45–49	21.5 ± 0.56	± 0.102	5.7 Positive

* D = actual difference between the two averages being compared.
PEd = probable error of the difference.

Difference between the two means is significant when $\frac{D}{PEd} > 3$. (See Sherman, H. C., Chemistry of food and nutrition, New York, 3rd edition, 1926, 604.)

** The periods were grouped in this manner in order to obtain greater accuracy by using larger numbers of cases. As observed from the chart there is little or no significant difference between the values for gross enlargement within the groups.

The weights of the heart and liver of some of the rats showing the greatest degree of renal enlargement on the "high protein" diet were compared with similar data for normal animals taken from Donaldson (1924). In no case was an increase in size of either of these organs demonstrable. This observation agrees with that reported by Osborne, Mendel, Park and Darrow (1923), and with many unpublished observations of these investigators.[2]

[2] Personal communication of Dr. Mendel.

Has the renal function after nephrectomy returned to normal before the final structural equilibrium has been reached or even when this stage is reached? Data bearing on this question have been published by Addis, Myers and Oliver (1924) who used nephrectomized rabbits as the experimental animals. They found that after 106 to 125 days when the *total* renal tissue was 66 per cent of that before nephrectomy the function as measured by the rate of urea excretion was 98 per cent of the normal. The values for rate of compensatory enlargement used by them were those of Hinman (1923) for rats. It seemed necessary to begin new experiments on renal function of rats under the conditions of the present study and the work is now in progress.

Finally, do these extremely large kidneys show evidences of tissue injury? The effect of diets rich in protein on the kidney is at present a favorite theme for debate. The material obtained from the present investigation should afford unique opportunity for such a study, the results of which will soon be published.

Complete protocols of all the experiments have been filed in the archives of the Wistar Institute, where they may be consulted.

SUMMARY.

The rate of compensatory enlargement of the remaining kidney after unilateral nephrectomy has been studied in adult rats fed diets containing various concentrations of protein.

A curve of enlargement on "standard" food (18 per cent casein) shows a rapid initial increase with subsequent slower rise to the 120th day. There is no significant difference between the value at 120 days (44 per cent) and that at 150 days (48 per cent).

A similar series with diets containing increasing concentrations of protein but with a constant time interval (21 days) after nephrectomy shows an increase in the degree of enlargement directly proportional to the protein content of the food. The values vary from 24 per cent with the 18 per cent casein ration to 77 per cent with the 90 per cent casein diet.

A third series shows the enlargement on "high protein" food (85 per cent casein). The values vary from 49 per cent at 3 days to 121

per cent at 150 days. There is no significant difference between the value at 120 days (123 per cent) and that at 150 days (121 per cent).

Determinations of total solids on the experimental kidneys show that the recorded enlargement involves mainly an actual tissue increase.

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A FILTERABLE YEAST-LIKE MICROORGANISM (SCHIZOSACCHAROMYCES FILTRANS, N. SP.).

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PLATE 7.

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INTRODUCTION.

The microorganism, the culture and significant qualities of which it is the purpose of this paper to describe briefly, has the interest of a type to those concerned with attempts to cultivate the filterable viruses. From an immediately practical standpoint it may well be regarded as a saprophyte and an accidental contaminant arising in a series of cultures primarily directed toward the study of the filterable virus of hog cholera.

From the beginning almost of our knowledge of filterable viruses as the cause of disease in man and animals it has been a debatable question as to how far filterability is to be regarded as signifying essential invisibility. It has come to be generally recognized that particles in order to pass the coarser earthen filters ordinarily effective for the removal of bacteria must have a smaller diameter of not to exceed one- or possibly two-tenths of a micron. Length, if combined with flexibility, need not be a limiting factor to the same degree. It would doubtless be granted that extensibility within reasonable limits must also be an important factor and that this might be influential in either permitting the passage of somewhat larger particles whose extensibility was great or preventing the passage of very rigid smaller ones. Naturally the state of agglomeration and the condition of the surface, whether adhesive or not, would be additional factors which might determine the actual ability of living particles of smaller dimensions than this to pass a filter. Optically, it is known that particles of about 1/10 micron or less in their longest diameter must appear

round under even the highest powers of the theoretically perfect microscope. Such particles unless characterized by some peculiar state of agglomeration or by some microchemical reaction must remain indistinguishable from inert particles of the same general size. Unrecognizable they would be essentially invisible.

Within these limits various well known examples may be mentioned. The filterable virus of pleuropneumonia of cattle, long cultivated, is visible without doubt, but admittedly pleomorphic, and this to a degree which combined with the factor of difficult visibility leaves a considerable latitude of uncertainty in the descriptions which have been given of it (1, 2). The virus of "*Hühnerpest*" propagated by Marchoux (3) and by Landsteiner and Berliner (4) was not seen by these authors. More recently described there are the visible and filterable *B. pneumosintes* of Olitsky and Gates (5), the essentially invisible virus of mosaic disease of Olitsky (6), and the likewise optically unrecognizable virus cultivated by Noguchi (7) from the tick *Dermacentor andersoni*. Considering the nature of their growth in culture these microorganisms may probably be minute bacteria. It may well be recalled here that Borrel (8) recognized that certain filterable saprophytic bacteria could be cultivated from water in his locality.

Borrel (9) also gave a brief account of a filterable microorganism from water which he regarded as a protozoan. Schaudinn (10) recognized that certain protozoa might have spirochete-like forms as a stage in a complex life cycle, and, as a suggested application of this postulate, indeed, predicted that yellow fever, of the virus of which it was at that time known that it was transmitted by a mosquito and that it was filterable, would be found to be such a spirochete. The actuality is that the *Leptospira icteroides* of Noguchi (11) is filterable by reason of its inherent properties but there is no evidence of its being a stage in the life cycle of any more complex parasite. These observations of Borrel and Schaudinn served to introduce into the literature of the subject the postulate that some of the filterable viruses might be very small stages in the life cycle of complex parasites, other stages of which might be clearly visible. The thought recurs in the reviews of the subject but concrete examples are so far lacking. Nearest to fulfilling the conditions comes the case of the trypanosomes. Novy (12) described cultures of these protozoa as becoming very small under cer-

tain conditions so that they could pass filters which had been ground thin, and more recently Reich (13) has found that the liver and spleen of animals infected with *Trypanosoma brucei* may frequently yield infectious filtrates. While these observations do relate clearly to protozoan parasites, yet they scarcely apply to the question in hand since there is little or nothing to suggest that the trypanosomes as a group are cyclic in constitution. The culture now in hand, which is in all probability to be classified as a yeast or yeast-like fungus, seems to fulfill quite perfectly the requirements of this very general postulate.

OBSERVATIONAL.

In connection with the study of hog cholera virus it was thought to apply to its cultivation a method developed by Gates (14) during the study of *Bacterium pneumosintes*. Collodion sacs were sealed to a glass tube 1 cm. in diameter and 14 cm. in length (ordinary bacteriological test-tubes with the bottom cut off). These were fitted into larger test-tubes in such a way as to project through and be supported by a tight cotton plug of the latter. The tube with sac attached was also plugged with cotton. Water was put in the inner tube to the height of 10 cm. or more and in the outer tube to cover the collodion, and the whole sterilized in the autoclave. The appliance is then, two tubes, an inner and outer, the fluid in which is separated by a collodion sac 1 cm. in diameter and about 2.5 cm. in length. Either may be separately treated as a bacteriological culture tube.

The experiments in question consisted in replacing the water in the inner tube with mixtures of nutrient bouillon, blood serum of either rabbits or swine, and adding pieces of fresh kidney of either rabbit or swine. A commercial hog cholera virus (unfiltered) was diluted 1/100 with sterile water and the outer tubes of a number of these preparations were inoculated with 1/10 or 2/10 cc. of this. The tubes were then incubated in various ways, aerobically and anaerobically, at room temperature and at 37.5°C., in all possible combinations. A number of these tubes developed growth of ordinary bacteria within a few days and were discarded. Others remained perfectly clear and limpid for weeks and were likewise eventually discarded. A few of the tubes after a week's incubation aerobically at room temperature or 37.5°C. showed a very slight but definite turbidity which remained, but did not increase to the point of sedimentation. Transfers to other similar tubes did not develop. All of these tubes sooner or later developed growth of easily visible microorganisms and were discarded, it being considered that the repeated opening of the rather cumbersome tubes rendered eventual contamination inevitable.

As transfers were made, samples for microscopic examination were reserved in small test-tubes (agglutination tubes) and these were kept on the desk. They were repeatedly examined both in hanging drop and by the application of various

staining methods. There was from the first a reasonable doubt as to whether the particles responsible for the turbidity, which could be clearly seen in hanging drop, might not be living. The reexamination remained a matter of interest for weeks and in the meantime the fluid gradually evaporated. It was several times restored to approximately the original volume with sterile distilled water. Some few days after such an addition of water three tubes in quick succession developed a much greater turbidity with some tendency to the formation of a flocculent precipitate easily redistributed by shaking. This was about 10 weeks after the segregation of the samples and approximately 3 months from the planting of the original cultures. The tubes were now found to contain undoubted microorganisms. So much detail has been given in order to make plain the uncertainties which surround the actual source of the culture.

The slightly turbid samples as segregated from the original tubes and in the succeeding weeks showed moderate numbers of particles, ill defined in optical character. They ranged from those barely visible, through a considerable number just clearly visible, to a small number which may have been 0.5μ in their larger diameters. The smaller particles appeared to be perfectly round; those somewhat larger also were round, and while usually single were sometimes in pairs. The largest of the particles were sometimes round but were often definitely angular suggesting the longitudinal section of an irregular but generally ovoid body. All the particles had active Brownian motion and the larger ones presented a definite halo.

Stained with methylene blue, even with heat or for a long time, and after various methods of fixation, nothing could be seen of these particles. When preparations were fixed in methyl alcohol and stained with full strength carbolfuchsin for from 3 to 5 minutes, or when Giemsa stain was applied, stained particles showing a general correspondence to those seen in the fresh were easily made out, but they presented no qualities sufficiently definite to distinguish them with certainty from precipitated stain or stained detritus. If, as is possible, there was any gradual development of more definite forms such as later appeared suddenly in abundance, it passed unnoticed in spite of repeated examinations.

Examination of hanging drops immediately the sudden increase in turbidity was noticed revealed very considerable numbers of a yeast-like microorganism. This was small as compared to the usual yeast, presenting as characteristic forms more or less round bodies about

1.5μ in diameter associated with longer or shorter structures of the type of abortive mycelia. The number of these was not sufficient to account for the turbidity and they were associated with many much smaller forms. Some of the constituents of the apparent mixture were very actively motile. It seemed impossible at first sight that there could be other than a gross contamination of the fluid but it remained of apparent interest to see whether the smaller forms presented might represent a more active multiplication of the forms previously seen. Suitable preparations were stained and it appeared that there was very little in the material stainable with methylene blue, while carbol-fuchsin and Giemsa stains quite adequately represented the picture presented by fresh preparations. Cultures made on the usual media emphasized the apparent peculiarities in that there was no immediate growth. The material was therefore subjected to a more systematic study with the following final results.

All of those agglutination tubes which showed the sudden increase in turbidity referred to yielded the same culture. The culture grows most consistently on horse blood agar at room temperature. Growth occurs at 37.5°C. but is slower and much less voluminous under these conditions. The addition of sugar to the blood agar neither favors nor interferes with the growth. On this medium even a rather heavy planting shows no change for the first 48 hours. On the 3rd day there may appear an alteration of the surface of the slant and examination with the glass reveals an abundance of very minute, discrete, rather moist colonies. Succeeding days render these visible to the naked eye and at the end of 3 weeks, if the planting has been thin, single, brownish white, mucoid colonies may reach a diameter of 2 to 3 mm. In case the planting has been heavy the covered surface presents a thick creamy mass of the same color. Touched with the wire the colonies are soft, easily spread, but never stringy. They are never soft enough to run down the surface. These appearances are dependent on the medium being freshly prepared, moist, and on the retention of moisture by at least partially closing the tubes. In the case of old medium, or that which has partly dried before the culture is luxuriant, the growth remains flat and dry, the colonies being small and discrete. Intermediate between these extreme alternatives there develop a few mucoid colonies on a surface generally covered with flat, dry,

discrete ones. Such cultures suggest a mixture, but transfer from any part of them to favorable medium restores the described luxuriant growth.

The blood in the agar shows no characteristic changes as growth develops. The condensation water of these blood agar tubes remains clear but gradually develops a heavy sediment. Scraped into distilled water, normal saline solution, or nutrient bouillon, the growth from the blood agar slant forms a homogeneous suspension with the greatest ease. This partly sediments in 2 or 3 days but is resuspended by slight shaking. The appearance (except color) and consistency of the fully devloped culture on blood agar resembles that of a luxuriant culture of ordinary yeast on the same medium.

The culture may be said to grow well on all of the usual culture media but the volume of growth attained on the other solid preparations used does not equal that on blood agar. Growth on media other than blood agar is also capricious in marked degree; that is, unless the culture from which the transfer is made is at the height of its vigor, the transfer is apt to fail or the resultant growth be very uneven. In bouillon with or without added sugar growth results in a flocculent or granular sediment tending to cling to the sides of the tube until dislodged by light shaking. There is sometimes a light ring at the top suggesting that growth takes place here and that the sediment results from the fall of this imperfect scum. When serum is added to the bouillon growth starts in the same way but the resulting sediment is much larger in amount and less granular. After several weeks there gradually develops a diffuse, heavy clouding throughout the fluid above the sediment reaching to the top and this clears but slowly over several weeks more.

Planted in a semisolid medium containing reduced amounts of bouillon constituents and with blood serum added (Noguchi's leptospira medium), heavy growth develops on the surface and in the upper few millimeters but does not extend below this.

Neither acid nor gas is developed in fermented bouillon to which Andrade's indicator and various sugars are added. Dextrose, lactose, saccharose, maltose, and mannitol have been tested. In the fermentation tube growth is confined to the open arm.

Growth on plain agar has always been scanty; on glycerol agar it

has sometimes been scanty and sometimes quite heavy, particularly in the lower part of the tube. On glycerol agar the growth has sometimes acquired a pale orange-yellow color with age.

Gelatin is not liquefied.

Litmus milk is not changed.

The microorganism has now been under observation for over a year. The growth characteristics as described have remained constant. The culture has been repeatedly plated and has been and is still apparently pure. Microscopically the culture has changed in that certain forms which were prominent in the early cultures are now more rarely seen. There was in the beginning a greater tendency than now for changes in the medium employed to result in a temporary predominance of particular morphological forms. The following description of the morphology of the culture is a composite based on the total experience with it.

Transfer of culture material which has aged to any medium or of a culture at the height of activity to an unfavorable medium results in a culture which for the first few days consists chiefly of what are at first sight simple micrococci of about 1.5μ diameter. These may be single and perfectly round; more frequently they are clumped in dense masses in which the individual cocci are only apparent at the edges; sometimes they are in pairs definitely lanceolated (Figs. 5 and 6). Cultures in plain bouillon, on plain agar or on blood agar somewhat dry when the plant is made, may develop no other forms than these. The micrococci are mostly stained rather faintly with methylene blue but some individuals are intensely stained. With carbolfuchsin or with Giemsa's stain they stain intensely. Neither in the fresh nor in the stained condition do the cocci exhibit any evidence of structure. As the cultures age the masses of cocci examined in the fresh state assume a definitely granular appearance. They now stain even less well with methylene blue. With Giemsa or fuchsin the granular condition is very evident, the particles being irregularly massed, interspersed with much poorly staining material, and varying in size from that of the original coccus to about 0.1μ. Every effort has been made to secure preparations which might exhibit some regularity of transformation of the cocci into the smaller granules, but without success. This might be but the rather usual granular transformation in old bacterial cultures but for the following facts.

If such a granular culture, or one consisting chiefly of cocci before they have changed, is washed into sterile distilled water and allowed to stand for 72 hours it is found to have changed completely. The masses of round cocci are no longer found in any considerable number and single round cocci are also rare. Ovoid cocci, single and in lanceolated pairs, of the general size of the original cocci, are now quite common and there are numerous forms of the same length (1.5μ) but thinner, so that they appear as definite bacilli (Fig. 7), and even more numerous are still smaller bacillary forms definitely grading down to the smallest visible ones (Fig. 10). Especially interesting are numerous instances in which the thinner bacillary forms appear as branches or buds from the ovoid cocci or the lanceolated pairs (Figs. 8, 9, 11, 12). Many of these definitely bacillary forms are very actively motile. They may exhibit this motility while attached to the larger forms and impart to the latter a very active movement although less rapid than that of the detached bacilli. With the dark-field microscope the very smallest bacillary forms can be recognized as frequently motile. The round or ovoid cocci are never motile in the absence of one of these recognizable buds or branches. The bacillary forms stain not at all with methylene blue and much less readily than do the coccoid forms with carbolfuchsin or Giemsa's method.

This complex result following the suspension of the coccoid culture type in distilled water is an essential restoration of the picture presented by the original samples after they had acquired a marked turbidity, as described in previous paragraphs. The same result is also spontaneously developed in the cultures, being characteristic of those colonies or masses which develop pronounced mucoid characters as likewise described above. There is a difference in that in the cultures the coccoid type is never so completely submerged as is often the case in the water suspension. A further difference is that the suspension of the culture in distilled water usually presents appreciable numbers of the larger yeast-like forms (Figs. 1 and 2) seen in the original material, while under cultural conditions favoring the mucoid transformation and the development of the motile bacillary forms the larger yeast forms are seldom or never seen.

The yeast-like forms appear in abundance under certain conditions which have not been brought under control. They have been some-

times, but are not usually, abundant in the condensation water of blood agar transplants during the first day or two before there is any visible growth. They have also been abundant and persistent in some of the glycerol agar cultures. Examined in fresh preparations when abundant these forms present the widest variety of shape. They are all resolved, however, into a coccus-like body, or clump of such, with a projected mycelial-like structure of variable length, width, and conformation. The mycelial projection frequently exhibits irregular contractions in the fresh condition, and usually does so when fixed and stained. The illustrations (Figs. 1 and 2) are from preparations left overstained. If differentiated the irregularities are emphasized and the projections are often coarsely granular, suggesting a disintegration into coccoid bodies. In the fresh, the mycelia are not granular and there is no suggestion of such a break-up. In the condensation water of blood agar cultures before visible growth appears there are frequently found clumps of coccoid bodies with a fringe of bacillary projections (Fig. 4).

As wholly aberrant there are rarely seen perfectly round bodies of larger size (5–6μ diameter). These in fresh preparation are homogeneous and thin walled, presenting no suggestion of a double contour or any heavier membrane. There are also seen rarely in stained preparations homogeneous, rather densely staining bodies of similar size and conformation. In a very few instances these have shown a suggestion of division into four ovoid bodies corresponding in size to the more usual coccoid forms.

The extreme pleomorphism presented by the original watery suspension and the earliest cultures naturally suggested a mixture. It was hoped to recover the smaller forms by filtration. The following experiment was done as a repetition of imperfectly executed preliminary trials.

Eight new Berkefeld filters, grade N, were prepared in the usual way. A number of cultures on blood agar 2 weeks old were suspended in distilled water and stood at room temperature for 5 days with frequent light shaking. The total quantity of suspension was about 100 cc. There was then added to this an equal amount of a heavy suspension of *Bacillus abortus* (Bang) made from an old laboratory culture capable of vigorous growth. This mixed suspension was immediately passed through the filters, 20 cc. through

each of the eight. The filtration was forced by the house vacuum and occupied about 10 minutes each. Each filtrate was divided immediately into two equal portions. One portion was planted on plain agar, 1 cc. per tube, the tubes sealed with wax, and incubated at 37.5°C. for 10 days as a test for *B. abortus*. The other portion was planted, 1 cc. per tube, on horse blood agar and kept at room temperature.

The experiment resulted as follows. Two of the filters passed *B. abortus* as well as the microorganism in question and are considered defective. Of the six shown to be efficient in removing *B. abortus*, the microorganism in question passed through three. Growth from the filtrates was slow, only developing after 2 to 3 weeks, and in only one case did all the tubes develop growth. In the other two cases about half the tubes developed. The growth which did develop was normal for the complete culture. All the forms which have been described were eventually observed in the first cultures from the filtrate. The small colonies consisting of cocci chiefly were the first to appear, but the mucoid transformation was prompt in all cases and was associated with the appearance of the motile bacillary forms. The yeast-like forms appeared when these first filtrate cultures were suspended in distilled water. This experiment was carried out when the microorganism had been under active cultivation for about 4 months.

In view of the possible origin of the culture in the virus blood of hog cholera its virulence for swine has been repeatedly tested. It does not produce cholera nor did the experiments develop any other evidence of pathogenicity.

DISCUSSION.

The culture described fulfills the condition postulated in the introduction. That is, we have to do with a pleomorphic microorganism certain forms of which are within the range of ordinary microscopic visibility, other forms at the limits of visibility, would, taken alone, be essentially invisible. Under certain conditions forms capable of regenerating the complete culture are filterable through Berkefeld N filters.

It follows as a practical consequence of this that in dealing with filterable virus material as the subject of cultivation experiments growths which are within the range of ordinary visibility may not be disregarded or discarded as inconsequential on this count alone.

The consideration of the systematic position of this microorganism is of considerable secondary interest. Because of the appearance of the larger forms referred to, the general character of the luxuriant growth, and the evident complexity of the cultural forms, I have considered that it was probably to be classified as a yeast or yeast-like fungus. The predominant type of multiplication in the earlier stages of all cultures and in all stages when cultural conditions are rather unfavorable is by fission. If, therefore, the assignment of the culture to the yeasts were accepted, it would fall naturally into the group of schizosaccharomyces.

The schizosaccharomyces as described by Guilliermond (15) seem to comprise two distinct groups at the present time. One of these is made up of a number of well known cultures closely related in fermentational activity, size, and general morphology to the yeasts whose direct multiplication is by budding. The other group comprises a number of microorganisms found in the organs of insects. They have been generally accepted as yeasts on morphological grounds but have not been cultivated. They are of smaller size on the whole than the first group and their systematic position would still seem to be open to some question. It is interesting that two of these, found in *Chermetis strobilobii* Kalt. and *Chermetis abietis* L., as described and classified by Sulc (16), are the smallest of the yeasts (length 1–2μ) and in this respect are comparable to the fission forms of the culture in hand. Considered to be a yeast the culture in hand appears to be more closely allied to these insect forms than to any other.

As an alternative to this assignment the microorganism above described might be thought of as a pleomorphic bacterium. The curious phenomenon of a non-motile micrococcus with motile bacillary branches would be presented, together with extreme changes in size in both the morphological series. The larger forms which have been thought of as yeasts might be reconciled with this view as involution forms. On this alternative the culture would have many points of resemblance to the group *B. radiobacter* of the root nodules of legumes and to *B. azotobacter* of the soil. With these bacteria extreme pleomorphism prevails and very small forms occur. Löhnis' (17, 18) attempts to recover the culture from filtrates were apparently unsuccessful in the case of *B. azotobacter*.

A second alternative has been constantly in mind, namely, that the culture may really be a mixture. This has seemed less probable as the properties of the culture have been developed. It involves the conception of at least two microorganisms both filterable and living in such close association that they are inseparable by plating methods. It need only be pointed out that even accepting this alternative it in no way diminishes the significance of the culture as a type of filterable microorganism presenting easily visible forms and readily cultivatable.

It may further be suggested that if this microorganism is a yeast the basic forms, small as they are, should show more evidence of definite internal structure than is the case.

While suggesting such restrictions and alternative interpretations as have arisen during the study of the culture, my inclination is to regard the microorganism as a fission yeast further characterized by its filterability and therefore designated provisionally as *Schizosaccharomyces filtrans*.

SUMMARY.

A microorganism cultivated in the course of experiments with the virus of hog cholera shows the following points of interest.

It is pleomorphic in extreme degree.

Among the forms are coccoid and bacillary bodies at the lower limits of visibility.

The culture can be completely regenerated after filtration through some Berkefeld N filters although these retain *Bacillus abortus* (Bang).

The culture is carried forward by multiplication of forms of easy visibility (1.5μ).

Associated with these at times are still larger forms bearing a striking general resemblance to those yeasts which develop abortive mycelia.

Motile bacillary forms appear to arise as buds or branches from round or ovoid cocci.

While cognizance is taken of possible alternatives the microorganism is provisionally classified with the yeasts and recognizing its filterability is named *Schizosaccharomyces filtrans*.

The culture is not pathogenic for swine.

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EXPLANATION OF PLATE 7.

FIGS. 1, 2, and 3. From 14 day culture on glycerol agar. Giemsa stain without differentiation. × 1620.

FIG. 4. From blood agar culture suspended in distilled water. Giemsa stain without differentiation. × 1620.

FIG. 5. From 2 day culture on blood agar. Carbolfuchsin stain. × 1620.

FIG. 6. Same culture as Fig. 5, stained with Giemsa's method. × 1620.

FIGS. 7, 8, 9, 10, 11, and 12. From suspensions of blood agar cultures in distilled water. Stained by Giemsa's method. × 1620.

Note.

The bacillary and branched or budded forms as shown in Figs. 7 to 12 are apt to be very actively motile.

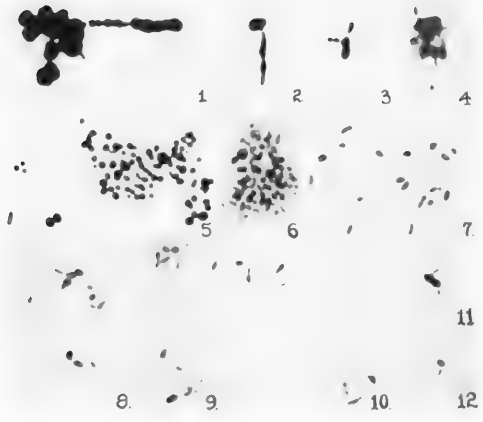
The independent division of the bacillary forms is strongly suggested in Fig. 7.

The origin of the bacillary from the coccoid forms is strongly suggested in Figs. 11 and 12, where the suspensions were active and the cocci free, as well as in Fig. 4 from a suspension of an old culture where the cocci remained in masses.

The granular transformation of the coccoid forms is shown in Figs. 5 and 6.

The yeast-like forms are shown in Figs. 1 and 2, while Fig. 3 from the same preparation shows how close may be the relation between these and the coccoid forms of Figs. 11 and 12.

An attempt to construct a complete developmental cycle on the basis of these suggestions seems to be unwarranted because of an insufficient understanding of what happens among the smallest forms of Figs. 5 and following.



(Lewis: Filterable yeast-like microorganism.)

THE HISTOLOGICAL EXPRESSION OF THE NATURAL RESISTANCE OF RABBITS TO INFECTION WITH HUMAN AND BOVINE TYPE TUBERCLE BACILLI.

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The separation of mammalian tubercle bacilli into two types, designated respectively the human and the bovine in accordance with their usual derivation, as first made by Theobald Smith depended primarily on a constant difference in virulence. The recently isolated cultures of bovine type in minimal doses cause a progressive, usually fatal tuberculosis when reinoculated on young cattle or on rabbits. In equivalent doses cultures of the human type cause only local, non-progressing lesions in these animals, lesions which frequently heal entirely. Smith later described differences in the progressive changes in the reaction curve when the type cultures are grown on glycerol broth of appropriate constitution, and also other less definitely measurable cultural distinctions were clearly drawn.

The foregoing statements of fact may be paraphrased with the reaction of the animal as a primary consideration. We would then say that cattle and rabbits have a high degree of natural immunity or resistance against infection with tubercle bacilli of the human type and are very susceptible to infection with those of bovine type. This natural immunity toward bacilli of the human type is by no means absolute. In the case of the rabbit the kidneys frequently become the seat of a slowly progressive local disease which eventually destroys their capacity to function and leads to the death of the animal. At times also quite extensive loci of disease develop in the lungs. The restraint is, however, sufficient to prevent a generalized spread of the infection, and death when it occurs is caused by interference with some local function.

The nature of this natural immunity is quite unknown and is a subject of considerable interest. It is known that the injection of human tubercle bacilli intravenously does at times lead to the formation of miliary tubercles in the lungs and that these must subsequently be reabsorbed to account for the generally normal condition of these organs when the animals are killed 2 or 3 months after infection. The resistance does not therefore depend on a total failure of the tissues to react to the presence of the bacillus. Nor can the natural resistance have all the qualities of the acquired immunity possessed against reinfection in greater or less degree by all tuberculous animals which have been infected for several weeks. It is generally considered that hypersensitiveness as manifested by a hyperactivity of exudative and destructive processes is in considerable measure responsible for this induced partially immune condition. The hypersensitiveness is lacking, of course, in the normal animal and takes as a matter of fact 2 or 3 weeks to develop its effectiveness. It must be assumed that this feature is lacking in the early days of a primary infection and can be at most a reinforcing factor in any expression of natural resistance. Beyond these two points, the certainty of some positive reaction to the human bacilli and the certain absence of the hypersensitive factors in the earlier stages at least of this reaction, present knowledge fails us.

As a preliminary to a further study of this matter we have carried out a comparison of the reaction of the lung of the rabbit to the two types of bacilli using histological methods. The purpose of the present paper is to report these observations.

Krause[1] outlines a somewhat similar study as being then in progress in his laboratory. His comments, directed wholly to problems related to the localization of the infection, make no reference to the comparative aspects of the study and do not suggest any particular reference to the question of immunity.

EXPERIMENTAL PART.

Fifteen rabbits of approximately the same weight and age were injected intravenously with 1 mg. of a culture of living human tubercle bacilli, and the same

[1] Krause, A. K., *Am. Rev. Tuberc.*, 1922–23, vi, 1.

number with 1 mg. of a living bovine strain. At 2 day intervals an animal of each series was killed, tissues being preserved in formalin and Zenker's fluid. Sections were cut in paraffin. Staining was done with the eosin-methylene blue method after Mallory and Wright and for tubercle bacilli by the Ziehl-Neelsen method. A description of the conditions found in representative individuals of each series on microscopic examination follows. The gross appearances were not informing.

The human type culture (No. 7156) used was a typical one isolated from sputum at the Henry Phipps Institute of the University of Pennsylvania in the fall of 1922. It has been cultivated on glycerol agar with monthly transfers. The virulence for guinea pigs is normal for the type.

The bovine type culture (Bovine xiv) was isolated from a cervical abscess occurring in a 9 months old calf in May of 1919. The location and character of the lesions were rather atypical. The material was injected into guinea pigs and cultures isolated from them on egg medium in the usual way.

After four monthly transplantations on the egg medium the culture was placed on glycerol agar and has since been maintained on this medium. The growth characteristics and virulence for guinea pigs and rabbits are characteristic for the type.

Rabbit 1.—Human bacilli injected. 2 days. There is much thickening of the interalveolar septa very irregularly distributed. This is partly due to a very intense capillary congestion and stasis, in part to an exudation of mononuclear and polynuclear leucocytes. The mononuclear cells are of varying size and conformation, chiefly large cells of "endothelial" type. Small lymphocytes are rare, and definite phagocytic macrophages are not seen. The polynuclear cells are chiefly of eosinophilic type, showing large granules rather indistinct in outline with a tendency to fusion. These cells are found especially in foci about the smaller arterioles, sometimes in small collections in capillaries and venules. The alveoli are generally empty of either cellular or serous exudation. Occasionally one is found to be filled with an exudate chiefly made up of large mononuclear cells. The smaller bronchial branches are frequently filled with similar cells showing that while, as measured by the alveoli affected, the exudation to the surface is slight, yet "*in toto*" it is significant.

The peribronchial lymphoid tissue is not large in amount. About the smaller divisions it shows some congestion in the peripheral zone and a slight increase of large mononuclear cells toward the center of the mass. A small amount of cellular debris is found in this region also. In connection with the larger bronchial branches lymphoid nodules are found between the cartilaginous plates and the mucosa, which are swollen, have broken into and in one instance through the mucosa.

A particular search for mitotic figures in those places where large mononuclear cells are collected in foci, both in the interalveolar septa and in the lymphoid tissue, failed to reveal any.

Tubercle bacilli were found in small numbers in the peribronchial lymphoid tissue. They appeared to be extracellular. None were found in other places.

Rabbit 4.—Human tubercle bacilli injected. 8 days. The interalveolar septa are much thickened and folded decreasing appreciably and generally the area of the alveoli. The thickening is due chiefly to an exudation of large mononuclear leucocytes. A moderate number of polymorphonuclear leucocytes are found. There is no congestion.

At widely scattered points the thickening of the septa is more massive and one or more alveoli are obliterated. In a few instances at these points there is organization into definite tubercles. In these cases the central cells are mononuclears, more uniform in character than in the exudate generally. In some cases they are of moderate size with basophilic cytoplasm; in others they are larger and both cytoplasm and nuclei stain rather faintly. None show any intense staining with eosin or other evidence of necrosis.

Eosinophil leucocytes are found occasionally in the exudate generally. They are very numerous in the lymphoid tissue about the arteries and arterioles. In one instance an arteriole is thrombosed, the cells of the thrombus being large mononuclears and eosinophils. The eosinophils are found in the muscular coat of this vessel as well as massed in its neighborhood.

Mitotic figures occur occasionally in the large mononuclear cells of the exudate generally. They are found in moderate numbers in the lymphocytes of the peribronchial lymphatic tissue.

Tubercle bacilli are found in moderate numbers in those portions of the interalveolar septa where the formation of tubercles is evident or suggested, but not elsewhere.

Rabbit 7.—Human bacilli injected. 14 days. The general description given of Rabbit 4, the 8th day animal, might well be applied to this animal. The following points of difference are to be noted. The tubercles are on the whole somewhat larger and some of them have a definite peripheral zone of lymphocytes. Tubercle bacilli are found in smaller numbers than in the 8th day animal, and like that one, only in the more central portions of the tubercles. The proliferative process, as evidenced by mitotic figures in large mononuclear cells in the interalveolar septa and in the tubercles, is still active. Eosinophil leucocytes are found but in much smaller numbers than previously.

Rabbit 11.—Human bacilli injected. 22 days. In the gross, there are minute, translucent, non-caseous tubercles scattered over the surface of the lungs. The organ is not definitely more firm to the touch than normal. In general the microscopic picture presented by this animal closely resembles that of the 8th and 14th day ones in this series. The interalveolar septa are markedly thickened, perhaps slightly less so than in the preceding animals. Mitotic figures are not found in them.

The tubercles are slightly more numerous in this animal. They are always single. The centers of the larger ones are almost unstained when viewed with

the low power and have a glassy appearance in some places. The high powers show the nuclei faintly outlined, the cytoplasm hyaline, the whole having taken very little stain. The outlines of individual cells can seldom be made out, small groups being fused and the mass apparently cracked and channeled (perhaps due to fixation shrinkage). The hyalinization process is found to extend in streaks along columns of cells to the periphery of the tubercle in numerous places. This is not accompanied by any crowding of the remaining cells, the consequence being that the whole tubercle is appreciably less in its content of well preserved cells than in the earlier animals. The well preserved cells are mostly large mononuclears. Occasional large phagocytes of the macrophage type are seen. There is a small amount of free cellular debris.

The lymphoid tissue is not prominent. One peribronchial lymphoid mass contains a definite tubercle which is hyalinized as just described. In other places the lymphoid tissue shows aggregates of large mononuclear cells.

Mitotic figures are found in large mononuclear cells in the peripheral portions of the tubercles in small numbers.

Eosinophils and amphophil polynuclear leucocytes are found scattered singly in the septa, in the peripheral zones of the tubercles, and in the lymphoid aggregates. Masses or clumps of these cells are not found.

Tubercle bacilli are found in very small numbers in the centers of the tubercles.

Rabbit 15.—Human bacilli injected. 30 days. The interalveolar septa in the subpleural regions are definitely thickened but in a very irregular way. In small patches this thickening is sufficient to obliterate alveolar spaces, in others it amounts to but the presence of an extra layer of mononuclear cells in the septum. The patches are rather definitely most marked beneath the pleura and in the regions about the vascular trunks fading through the less thickened septa to normal lung tissue in the midzone. Definite tubercles are found both in the subpleural region and in the region about the main vascular trunks. In the less thickened septa there are found small collections of large mononuclear cells more or less interspersed with lymphocytes. Occasional giant cells quite isolated from other cell collections are also found in this midregion.

The peribronchial and perivascular lymphoid tissue is scanty.

The tubercles, some of them large, consist almost entirely of large mononuclear cells. There is no definite demarcation into zones except for the suggestion of an imperfect capsular layer one or two cells thick. The cells are chiefly large mononuclears. Some of them are hyaline both as to nucleus and cytoplasm, staining very faintly. Others, perhaps the majority, are of normal appearance. The normal and abnormal cells are intermixed with no regular pattern. Occasional lymphocytes and a minimal amount of cellular debris are found among the mononuclear cells.

No mitotic figures were found and no tubercle bacilli.

Rabbit 16.—Bovine bacilli injected. 2 days. There is a very marked general thickening of the alveolar walls by an exudation consisting of large and small

mononuclear and polymorphonuclear cells. Many of the latter are of eosinophil type. The thickening of the septa is sufficient in many places to almost obliterate the alveoli by compression although the alveolar wall remains clearly defined and the alveoli generally are quite free of exudate. There is no marked congestion either generally or focally. The mononuclear cells of the exudate are on the whole rather large and faintly staining. The nuclei tend to take a violet tinge with eosin-methylene blue. There are scattered, rather large aggregations of mononuclear cells, with a moderate amount of cytoplasm, which retain a rather intense, clear blue with the stain. An occasional mitotic figure is found in the interalveolar septa, pertaining to cells of the large mononuclear series.

The lymphoid tissue about the bronchial vessels is greatly increased in amount chiefly by an increase in the size of the aggregates. Lymphoid cells undergoing mitosis are found in very large numbers in the peripheral zone of the nodules. The lymphoid tissue shows a scattering infiltration with large mononuclear and polymorphonuclear leucocytes.

Tubercle bacilli, taking the sections generally, are found in but small numbers and then in the interalveolar septa; none were found in the lymphoid tissue. In a few focal points—those previously described where the septa were most swollen —collections of large numbers of bacilli, single and in clusters were found. The arrangement of the clusters was such as to suggest the intracellular position of the bacilli.

Rabbit 19.—Bovine bacilli injected. 8 days. The interalveolar septa are generally much thickened and folded due to the excessive cellular content consisting of large mononuclear cells chiefly. Many polymorphonuclear cells are also found, eosinophils being more numerous than amphophils. Over considerable areas the thickening of the septa is of such extent as to nearly or quite obliterate the alveoli, the wall of the latter often remaining intact. In many places there is a very definite organization of the cellular content of the septa into tubercles and these are in various stages. Some are little more than collections of large mononuclear cells with a tendency to basophilic staining associated with a few amphophilic and eosinophilic polymorphonuclear leucocytes. In others similar groups of cells are partly surrounded by and merged in the periphery with lymphocytes. In a few places the mononuclear cells are massed closely and show decreased staining intensity. There is no definite necrosis. The tubercles are more numerous and somewhat further advanced than in the "human" animal of the same date.

The periarterial lymphoid tissue shows masses of eosinophils.

The peribronchial lymphoidal aggregates are of large size. In some instances their central portions almost to the periphery are occupied by large mononuclear cells. In these instances the central portions also contain small foci of lymphocytes, some polymorphonuclear leucocytes, and a moderate amount of cellular debris. In some instances also the submucosa and mucosa of the adjacent bronchial vessel are infiltrated with lymphocytes even to the surface.

Mitotic figures are abundant in large mononuclear cells in the interalveolar septa; in large mononuclear cells in the tubercles; in large mononuclear cells in the central portions of the lymphoid aggregates in those instances in which these cells are prominent there; and in the lymphocytes in the lymphoid tissue generally.

Tubercle bacilli, single and in clusters, are abundant in the central portions of the more advanced tubercles. They are found single and in small numbers in the peripheral portions of the tubercles among the lymphoid cells and in the peribronchial lymphoid tissue.

Rabbit 22.—Bovine bacilli injected. 14 days. In the gross, the pleural surfaces of all lobes are marked with minute, glistening, translucent, raised tubercles, giving the lungs a granular appearance. Some of these tubercles have coalesced. Organ is firm.

The interalveolar septa generally are somewhat less thickened than in the previous rabbits of the bovine series. Mitotic figures are no longer to be found in any number in the septa when these are definitely removed from well defined tubercles.

The tubercles are much more numerous than in the 8th day animal, the addition in numbers being apparently due in large part to the organization of new, small tubercles in contiguity with the older ones. The lines of demarcation while generally rather distinct are not always so. The larger tubercles have undergone a very definite central necrosis.

Eosinophilia is pronounced in the peripheral regions of the tubercles, in the interalveolar septa, and particularly in the periarterial lymphoid tissue.

Proliferation as evidenced by mitotic figures is still in evidence in the large mononuclear cells in the peripheral portions of the smaller tubercles and in similar cells in some of the lymphoid aggregates. No mitoses are found among the lymphocytes.

The lymphoid tissue shows some well defined tubercles. The lymphocytes are quite generally replaced by large mononuclear cells.

Tubercle bacilli are very abundant in the tubercles particularly in the central portions of the older ones. They are also abundant in those areas of the lymphoid tissue where the large mononuclear type of cell predominates.

Rabbit 26.—Bovine bacilli injected. 22 days. In the gross, the lungs are much enlarged, very firm to touch, and granular. The surface is heavily marked with translucent miliary tubercles most of which have gray centers.

Microscopically, the prevailing picture is that of conglomerate miliary tubercles, the majority of which show central necrosis. These occupy fully two-thirds of the lung. Between some of the tubercles there are large alveolar spaces containing a little exudate and cellular debris, while similar ones are entirely empty. The interalveolar septa where not in contact with tubercles are but slightly thickened.

The peribronchial and periarterial lymphoid tissue is involved in the tuberculous process which here is continuous with that in the adjacent lung structure so

that the lymphoid structure is no longer demarcated or clearly recognizable. Small collections of lymphocytes are scattered here and there in the tuberculous masses. These are largest in the peribronchial and periarterial regions. In these foci mitotic figures are readily found. Occasional mitoses are found in large mononuclear cells in the peripheral zones of the tubercles. Clusters of polymorphonuclear cells both amphophils and eosinophils are recognizable but are always fragmented and irregular in staining reaction.

The blood vessels and bronchial vessels contain much cellular debris.

Wherever in the section tubercle formation is definite or where groups of cells have undergone hyaline transformation tubercle bacilli are abundant. In the definitely necrotic centers of the tubercles they are present in enormous numbers. In the remaining, more normal interalveolar septa they are scanty for the most part or absent. They are also difficult to find in bronchial and blood vessels even where the contents of these are obviously necrotic cellular remnants.

Rabbit 29.—Bovine bacilli injected. 27 days. Microscopically, discrete and conglomerate tubercles dominate the picture occupying approximately four-fifths of the area of the section. Most of the recognizable cells in the tubercles are large mononuclears. The nuclei stain faintly and the cytoplasm staining also faintly has a hyaline appearance. Many of the tubercles show complete necrosis at the centers. Only in the peripheral zones of some of the tubercles are there small areas where the mononuclear cells stain normally. Even in these areas mitotic figures are not found.

The interalveolar septa adjacent to the tubercles are thickened by mononuclear cell increase. In the small areas which are distinctly removed from tubercles the septa are quite normal in appearance. The alveoli contain very little exudate of any kind.

The lymphoid tissue of the peribronchial regions is almost obliterated by the formation of discrete and conglomerate tubercles. Such remains as are seen show no mitotic figures or other evidence of activity.

The bronchial vessels contain a small amount of cellular debris.

Throughout the section, usually in the tissue between contiguous tubercles, there are found small collections of polymorphonuclear leucocytes. These are almost always in some stage of degeneration.

Tubercle bacilli are readily found within the tubercles and wherever these are necrotic, bacilli are found in large numbers. Outside tubercles bacilli are found with difficulty.

Comparative Description.

A comparison of the foregoing protocols, as well as those not printed, reveals a close similarity in the effects of the two types of bacilli up to the 8th day. The lesions during this period are primarily an increasing interstitial exudation of mononuclear and polymorphonuclear leuco-

cytes affecting the walls of the alveolar spaces. While more or less irregular, the distribution of this lesion may properly be said to be general and diffuse. It is remarkable that the rather extensive changes as revealed by the microscope have caused no definite macroscopic change. The alveoli are very free from an exudation either serous or cellular. Considering the extent or the quality of this interstitial inflammation by itself, and it is the dominant feature of the picture, there is no difference to be made out between the human and bovine series.

In the human series the interstitial inflammation persists with little change until about the 20th day and then gradually is reabsorbed. By the 30th day only traces of it remain in the midzonal portions of the lung, while in the subpleural regions and about the central main vascular trunks the thickened interstitial tissue, more irregular than before, is still plainly evident. In the bovine series the interstitial diffuse inflammation is apparently persistent, but as the tubercles develop gradually ceases to be the dominant feature in the picture. In the last animals of the series the alveolar walls where adjacent to tubercles are thickened, while those more distant are quite normal. This suggests that a great deal of reabsorption of the exudate has taken place in this series also.

Tubercle formation is evident in both series from the 2nd to the 4th day and is equally marked in every respect till the 8th or 10th day. From this point the two series diverge in an extreme degree. In the human series the tubercles enlarge somewhat up to the 18th day, but do not become more numerous, do not become conglomerate, and show no necrosis. After the 18th day the texture of the tubercles becomes looser, individual cells become somewhat hyaline, and the appearances generally suggest that they may be undergoing a reabsorption. By the 30th day they seem definitely less numerous. Those that remain are in some instances quite large and present ill defined connective tissue formation in the peripheral zone. In the bovine series after the 8th day the tubercles increase progressively in number, they coalesce to form conglomerate masses which undergo a process of fusion, and they become necrotic at the centers. Necrosis is evident at 14 days. These changes continue to develop to the end of the series when three-fourths to four-fifths of the area of the sections is occupied by clearly defined tubercles.

Tubercle bacilli are not abundant in either series up to the 8th day, and the two series show about equal numbers during this period. They are found by locating in the diffuse exudate those more definite clusters of mononuclear cells which seem precedent to the formation of well defined tubercles. Associated with those cells, sometimes within them, sometimes apparently between them, the bacilli are found.

After the 8th day bacilli become very difficult to find in the human series. In the bovine series they become increasingly numerous and very rapidly so. By the 14th day in this series, when tubercle formation is well advanced and necrosis has begun, the bacilli are abundant in the tubercle, and after this time are frequently found there in enormous numbers. Even in this series it is very difficult and usually impossible to demonstrate bacilli in the interalveolar tissue apart from all collections which are definitely segregated in such a way as to suggest tubercle formation.

The lymphoid tissue shows a marked hyperplasia in the bovine series from the 2nd day to about the 20th day. After this the lymphoid tissue becomes increasingly occupied by tuberculous tissue and finally almost disappears as such. Throughout this series frank development of tubercles in the lymphoid tissue is later than in the interalveolar exudate. In the human series the lymphoid tissue undergoes no definite hyperplasia, as estimated by the amount of tissue present, and does not become tuberculous to any considerable extent. Mitotic figures are frequently found in cells of lymphocytic type showing that there is here also an increased lymphoidal activity. Occasional large mononuclear cells associated with a few tubercle bacilli were found in lymphoid tissue early in the human series.

Experiments with Killed Bovine Bacilli.

As a check on the series of observations just described a shorter series was carried out in which 1 mg. of the bovine culture previously used was injected into two series of rabbits; in the one series living bacilli were used; in the second series the same suspension was used after heating to 60°C. for 1 hour, a procedure known to be lethal to the bacillus. Animals in each series were killed at 4 day intervals and examined in the same manner as that described.

The series injected with living bacilli developed in all respects in a comparable manner to that of the previous bovine series.

In the series injected with the killed bacilli the diffuse interstitial exudation developed with much the same intensity as that described for the living cultures either bovine or human. This exudate persisted in a way comparable to that previously observed in the human series, being still well defined at the 20th day which was as far as the series was carried. Well defined tubercles were not formed, but in the earlier members of the series small clusters of large mononuclear cells were segregated and with these were sometimes found a few tubercle bacilli. Polymorphonuclear cells were much less abundant in the exudate with killed bacilli than in the case of the living, either human or bovine. They did occur as collections of eosinophils chiefly and for the most part localized in foci of considerable mass in the perivascular tissues, particularly in the peribronchial submucosa. The lymphoid tissue shows an indefinite hyperplasia in this series marked by an increase in mitotic figures in the small lymphocytes within the nodules, but with no definite increase in the size and number of the nodules.

DISCUSSION AND SUMMARY.

From the foregoing description it is evident that when rabbits are inoculated intravenously with equal amounts of tubercle bacilli of bovine and human type respectively, they are subject to an immediate reaction in the form of an interstitial pulmonary exudation, which, being of equal severity and character does not serve to distinguish the type.

There is an hyperplasia of the lymphoid tissue which is much more pronounced in the bovine series and which may distinguish this type. Opinion on this point may well be reserved until other typical cultures are examined for this response.

The two types are sharply distinguished by the behavior of the tubercle bacillus and by the progression of tubercle formation. Tubercles are formed by both types and for about 1 week after inoculation they are not distinguishable. Progressively thereafter those formed as a response to the bovine bacillus become more numerous; they caseate and become conglomerate, finally coming to occupy the major part of the pulmonary tissue and its associated lymph nodes.

With the human type the tubercles do not progress to caseation, do not become more numerous after their first well defined formation, and finally tend to disappear. The human type bacillus does not multiply considerably, if at all, and disappears early. The bovine bacillus suffers little or no restraint in growth and finally multiplies enormously. It seems clear that so far as histologic evidence goes the fundamental difference in the reaction of the rabbit to the two types of tubercle bacilli is referable to the ability of the animal to restrain the growth of the human type or to the prevalence of conditions which permit a most vigorous multiplication of the bovine type. The initial cellular responses seem to be qualitatively of the same order and their quantitative distinctions are for the most part developed coincidently with the manifest growth of the bovine type bacillus.

If we undertake to state the observed results in the terminology of immunity we can say only that the histologic picture discloses a difference in the rate of bacillary multiplication which suggests that a difference in the physiologic requirements for growth of the two types of bacilli is satisfied or unsatisfied, in the respective cases, by the rabbit as host; or on the other hand, that there is a positive growth-restraining action exerted with efficiency against bacilli of the human type. It is evident that the present observations furnish no points of discrimination between these alternatives. There is, however, an occasional result of the injection of human type bacilli into rabbits (not seen in this series) which offers a suggestion.

When animals so injected are allowed to live for 2 or 3 months, the lungs at autopsy not infrequently present a few nodules of large size, often 1 cm. in diameter, which are found to be well encapsulated, soft, caseous masses. These often contain large numbers of tubercle bacilli. Since we know nothing of the particular conditions which give rise to these rather exceptional formations it is impossible to draw general conclusions from them, but they do suggest that the rabbit is not lacking in the food materials required by the human type bacillus; and that if the more usual suppression of this type is due to failure of its essential nutritives, it is rather a question of the distribution within the animal than an absence which is responsible. The usual result would then appear to be due to a positive growth-restraining action exerted against the human type bacillus.

Certain other points of interest in the histologic picture described are worthy of comment.

The lymphocytes do not appear as active cells in any preponderant way in either series and they are much less in evidence in the immune case (human type) than in the non-immune (bovine type). This might suggest that the activity of this cell type is a response to infection rather than that it furnished an effective preexisting barrier against infection in this particular case. If the lymphocytes were the most important agents in the immune reaction, it might be expected that they would show an immediate sharp response in the human series.

The large mononuclear type of cell is clearly most closely related physically to the tubercle bacillus within the body of the rabbit and this without distinction as to bacillary type. Foci of these cells are the loci of the disappearing bacilli of human type, and in either the active or necrotic state similar cell collections are the site of the most vigorous multiplication of the bovine bacilli. These cells undoubtedly stand in a central position in any picture which can be drawn of experimental tuberculosis in the rabbit and deserve as a consequence all of the very considerable attention they have received at the hands of numerous observers in recent years.

It has been quite usual of late to consider that the whole of the essential reaction of the animal against tubercle bacilli is carried by the cells of the mononuclear series, either lymphocytes or large mononuclears according to the predilections of the observer. We cannot, however, entirely overlook the presence in very large numbers of polymorphonuclear leucocytes, both amphophilic and eosinophilic, in this experimental series. They are much less prominent in the animals injected with the killed culture and hence can hardly be neglected on the assumption that they are merely a part of a reaction to an indifferent foreign body They are in large measure a reaction to the living organism: whether a primary and direct or a secondary, indirect consequence of its presence we are unable to decide.

These cells are not massed in any regular relationship to the well formed tubercles or to the clusters of mononuclear cells initiating tubercle formation. They are also very much less abundant in the very severe late lesions of the bovine type where enormous numbers of

bacilli are enclosed in the tubercles. It seems possible that the polymorphonuclears are a response to the living free tubercle bacilli as contrasted with either the dead bacilli or the living bacilli segregated in mononuclear cell clusters or in tubercles. They would appear also to be related to something apart from the bacillus itself, either a diffusion or disintegration product, since phagocytosis of bacilli, or the presence of bacilli in close physical relation to polymorphonuclear leucocytes, is so infrequent in general as not to have been observed in this series of experiments.

CONCLUSIONS.

The natural resistance of the rabbit to infection with the tubercle bacillus of human type is apparently referable to a failure of this type of bacillus to multiply in the body of this species to any considerable extent. Exceptionally there are localized lesions (kidneys and lung nodules) which are associated with abundant growth and which show that this failure to multiply may be due to some positive growth-restraining factor rather than to a failure of suitable nutritive materials. There is no qualitative difference in the cellular response to the bacillus of human type contrasted with that to the bovine type, which seems adequate to account for the difference in susceptibility. The eventually more abundant tubercle formation and the degenerative changes occurring in the tubercles formed with the bovine type bacillus seem to be a consequence of the abundant growth of this type; the initial response to equal quantities of the two when injected intravenously being approximately the same both quantitatively and qualitatively.

EFFECT OF AGE ON SERUM LIPOIDS AND PROTEINS.

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Blood serum is endowed with the property of inhibiting the multiplication of fibroblasts and epithelial cells, as has been shown by the experiments of Carrel and Ebeling.[1] It was also found that this growth-restraining power markedly increases with the age of the animal from which the serum is taken.[2] This phenomenon is due chiefly to the lipoids of the serum and also in some measure to the proteins.[3] However, serum also contains certain principles which precipitate with the euglobulin and possess a slight activating effect on the growth of connective tissue cells.[4] In old age, this property of the serum euglobulin was found to decrease.[5] The growth-restraining power of serum in the senescent period is probably due to some change in its lipoids or its proteins, or in both. The experiments reported in this paper were designed to test this supposition, and to determine the nature of the modifications that occur in the protein and lipoid constituents of serum in the course of life.

EXPERIMENTAL.

Sera were obtained from 6 month old chickens and from 4 to 5 year old hens of the same breed. The lipoid and protein fractions were separated and tested for retarding action on the growth of fibroblasts, the lipoid of the serum of the young animal being compared with

[1] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317; 1922, xxxv, 647; xxxvi, 399; 1923, xxxvii, 759.

[2] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599; 1922, xxxv, 17.

[3] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1925, xlii, 143; *Compt. rend. Soc. biol.*, 1925, xciii, 79.

[4] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxvii, 653.

[5] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxviii, 419; *Compt. rend. Soc. biol.*, 1924, xc, 172. Carrel, A., *Compt. rend. Soc. biol.*, 1924, xc, 1005.

that of the old hen, and the serum protein of the young chicken with that of the old one. The sera were also analyzed for the concentration of various proteins and lipoids.

Method of Preparing the Lipoid and Protein Fractions.

The sera were poured drop by drop, with constant stirring, into three times their volume of 95 per cent alcohol previously cooled to −10°C., and allowed to stand for 2 hours in a freezing mixture. The precipitated protein was centrifuged and washed twice with 95 per cent alcohol, three times with absolute alcohol, twice with a mixture of absolute alcohol and absolute ether, and finally three times with absolute ether. The alcohol and ether had been previously cooled to −5°C. or below, and the protein was separated from them by centrifuging in a tube surrounded by freezing mixture in a brine-cooled centrifuge, so that the temperature did not rise above zero degree throughout the process. The protein fractions of the sera were immediately dried *in vacuo* over sulfuric acid and redissolved in Tyrode solution. As only a very small amount of the protein became insoluble, solutions could usually be obtained equal in protein concentration to that of the original sera. Nitrogen determinations were made and the protein concentrations of the solutions adjusted to the same ratio as that of the original sera.

The alcohol in which the protein was precipitated and the alcohol and ether washings were mixed, filtered, and evaporated to dryness *in vacuo*. Shortly before the preparation of the cultures, the lipoid residue was emulsified in a volume of water equal to the original volume of serum, the pH adjusted, the cryoscopic point determined, and corrections for salt concentration were made.

Method of Testing the Growth-Inhibiting Action of the Protein and Lipoid Fractions.

The above preparations were tested on fibroblasts from a 14 year old strain, the two halves of the same piece of tissue being cultivated, one in the serum lipoid of the old animal and the other in the serum lipoid of the young animal. Since the two halves of the same culture of an old strain of fibroblasts grow at a uniform rate when the media are uniform,[6] any variation in growth would be due to differences in the media. The original sera were compared with each other in the same way, and also the protein fractions from the sera of the old and young animals. The technique described by Ebeling[6] for measuring the relative growth of the tissues was used. The original area and the area after 48 hours incubation were measured, and the rate of growth was calculated by dividing the latter by the former. The culture media consisted of 1 part of plasma, 1 part of either lipoid emulsion, protein solution, or serum, and 1 part of lipoid emulsion, protein solution, or serum containing 5 per cent embryo juice.

[6] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

Effect of Protein and Lipoid Fractions on the Growth of Fibroblasts.

In every case (Table I), the serum lipoids of the 4 year old hens were more inhibiting to growth than those of the 6 month old chickens. The same is true of the serum proteins (Table II) and also the total

TABLE I.

Comparative Rate of Growth of Fibroblasts in Serum Lipoid from Old and Young Chickens.

Group No.	Culture No.	Chemical preparation No.	Serum No.		Rate of growth in lipoid emulsion		Ratio of growth: Young / Old	Average ratio
			Young	Old	Young	Old		
1	2414 A	X-144	1431	1430	6.30	5.80	1.10	
					7.20	5.50	1.30	1.25
					3.80	2.80	1.35	
2	2781 A	X-232	2230	2229	3.48	2.64	1.30	
					5.40	2.90	1.85	1.65
					3.85	2.15	1.80	
3	2799 A	X-236	2402	2401	3.45	3.00	1.15	
					3.08	2.47	1.25	1.22
					2.90	2.30	1.25	
4	2823 A	X-241	2431	2432	4.00	3.20	1.25	1.28
					4.10	3.20	1.30	
5	2841 A	X-246	2494	2495	8.30	5.70	1.45	
					5.60	4.10	1.35	1.33
					8.40	7.00	1.20	
6	2857 A	X-249	2528	2529	5.90	4.70	1.25	
					7.20	4.35	1.65	1.53
					10.20	6.01	1.70	
Average.....								1.38

sera (Table III). Hence it is evident that changes take place with age in both the lipoid and protein fractions of the serum of an animal, which cause its increased inhibiting action on the growth of fibroblasts. The average ratio of the growth in the serum lipoid of the young animal to that in the serum lipoid of the old animal was 1.38, while the

ratio of the growth in the young serum to that in the old was 1.56. The ratio for the protein fractions was 1.50. While there is a wide variation in the figures from which these average ratios were obtained, due to individual differences in the animals and to the inevitable ex-

TABLE II.

Comparative Rate of Growth of Fibroblasts in Serum Protein from Old and Young Chickens.

Group No.	Culture No.	Chemical preparation No.	Serum No.		Rate of growth in serum protein		Ratio of growth: Young / Old	Average ratio
			Young	Old	Young	Old		
1	2413 A	X-145	1431	1430	7.60	4.40	1.70	
					6.60	5.10	1.30	1.43
					10.00	7.60	1.30	
2	2774 A	X-233	2230	2229	6.65	5.00	1.30	
					8.60	6.90	1.25	1.25
					7.00	5.70	1.20	
3	2800 A	X-237	2402	2401	4.90	3.00	1.60	
					7.00	3.40	2.10	1.83
					6.70	3.70	1.80	
4	2822 A	X-243	2431	2432	4.50	2.60	1.70	
					4.00	3.20	1.25	1.45
					5.40	3.90	1.40	
5	2851 A	X-247	2494	2495	4.25	3.10	1.35	
					6.50	2.70	2.40	1.85
					8.27	4.65	1.80	
6	2863 A	X-250	2528	2529	4.20	3.25	1.30	
					6.00	4.90	1.20	1.20
					6.20	5.50	1.10	
Average								1.50

perimental error, the average ratio for the lipoid differs from that of the sera and protein fractions in a manner to be logically expected. It is known[5] that the sera of the young animals contain in addition to inhibiting substances a small amount of growth-stimulating substance precipitated with the euglobulin by CO_2. This stimulating substance

is absent from the sera of the older animals.[5] Thus, in Ebeling's experiments, the difference in rate of growth of fibroblasts in the CO_2 precipitate of sera of old and young chickens was 0.18.[5] The ratio 1.56 for the sera is due, then, to two factors, the smaller quantity of inhibiting substance in the sera of the younger animals, and the pres-

TABLE III.

Comparative Rate of Growth of Fibroblasts in Serum of Old and Young Chickens.

Group No.	Culture No.	Chemical preparation No.	Serum No.		Rate of growth in serum		Ratio of growth: $\frac{\text{Young}}{\text{Old}}$	Average ratio
			Young	Old	Young	Old		
1	1448 C	X-142	1431	1430	8.40	7.60	1.10	
					6.95	5.10	1.35	1.28
					9.30	6.50	1.40	
2	2773 A	X-231	2230	2229	7.10	3.50	2.00	
					5.70	3.35	1.70	1.80
					6.00	3.55	1.70	
3	2813 A	X-235	2402	2401	4.50	2.88	1.55	
					8.90	4.25	2.10	1.62
					7.95	6.70	1.20	
4	2802 A	X-242	2431	2432	5.30	3.90	1.35	
					6.00	3.80	1.60	1.40
					8.60	6.90	1.25	
5	2842 A	X-245	2494	2495	13.90	7.20	1.90	
					9.40	6.30	1.50	1.65
					7.90	5.10	1.55	
6	2873 A	X-252	2528	2529	4.80	3.54	1.35	
					9.75	4.58	2.10	1.63
					5.40	3.74	1.45	
Average.....								1.56

ence of a stimulating substance. Since the latter would be absent in the lipoid fractions, a lower ratio for these would be expected and was actually found.

The data given here do not show how much greater is the inhibiting action of the lipoid than that of the protein fraction, since the lipoid

and the protein were not tested on the two halves of the same tissue, and the growth of two different pieces of tissue cannot be compared, because of the marked effect of their previous culture medium and their inherent growth activity. The relative effect of the lipoid and protein was demonstrated in previous experiments,[3] in which the lipoids proved even a little more inhibiting than the sera from which they were derived, while the protein fractions were much less inhibiting than the original sera. It is interesting to note that although the protein is less inhibiting than the lipoid, the rates at which their inhibiting action increases with age seem approximately equal.

TABLE IV.

Concentration of Protein in Sera of 6 Month and 4 to 5 Year Old Chickens.

6 mos. old			4 to 5 yrs. old		
Serum No.	Per cent nitrogen	Per cent protein	Serum No.	Per cent nitrogen	Per cent protein
1431	0.455	2.85	1430	0.885	5.53
2230	0.462	2.89	2229	0.690	4.32
2402	0.621	3.88	2401	0.912	5.70
2431	0.634	3.96	2432	0.707	4.42
2494	0.670	4.18	2495	0.547	3.42
2528	0.532	3.33	2529	0.709	4.44
Average...	0.562	3.52		0.742	4.64

Concentration of Protein and Various Protein Fractions in Sera of Old and Young Animals.

The sera used for the growth experiments were tested in the course of the work for their concentration of protein (Table IV). Here again, the individual variations are large, but it is evident that in general the sera from the older animals contain a higher concentration of protein, the average being 0.742 per cent nitrogen for the 4 to 5 year old hens, and 0.562 per cent nitrogen for the 6 month old chickens. The ratio of the average concentrations was 1.32. This corresponds fairly closely to the ratio that represents the inhibiting action on growth, *i.e.*, the rate of growth in the protein fractions is approximately inversely proportional to the protein concentration. Since serum contains a growth-stimulating substance which precipi-

tates with CO_2, a determination of the concentration of protein not precipitated by CO_2 was made on sera from old and young chickens. Here again, the concentration was found to be greater in the sera from the older animals (Table V). Hence, it is evident that the

TABLE V.

Concentration of Various Protein Fractions of the Sera of Old and Young Chickens.

Serum No.	Per cent total protein	Per cent protein not precipitated by CO_2	Per cent protein precipitated by CO_2. By difference	Per cent globulin in CO_2 filtrate	Per cent albumin
3 to 5 mos. old					
3007	3.13	2.92	0.21	1.45	1.47
3020	3.22	2.50	0.72	1.21	1.29
3029	2.29	1.87	0.42	0.56	1.31
3036	3.04	2.53	0.51	0.94	1.59
3040	2.52	2.06	0.46	0.90	1.16
3046	3.56	2.26	1.30	1.13	1.13
3053	3.28	2.76	0.52	1.42	1.34
3064	3.31	2.66	0.65	1.35	1.31
Average.....	3.04	2.45	0.60	1.12	1.33
4 to 5 yrs. old					
3006	4.89	3.19	1.70	1.58	1.61
3021	2.99	2.41	0.58	1.00	1.41
3030	4.89	3.69	1.20	2.37	1.32
3035	4.12	3.15	0.97	1.31	1.84
3039	4.55	2.20	2.35	1.17	1.03
3045	3.63	2.53	1.10	1.25	1.28
3052	3.94	3.14	0.80	1.45	1.69
Average.....	4.14	2.90	1.24	1.45	1.45
Ratio: $\frac{\text{Old}}{\text{Young}}$	1.36	1.18	2.06	1.29	1.09

increase in the inhibiting action of the protein with age is due not only to the disappearance of the small quantity of stimulating substance in the CO_2 precipitate, but also to a change in the concentration of inhibiting protein.

Determinations were also made of the concentration of albumin

and of pseudoglobulin[7] remaining in the filtrates from the CO_2 precipitation. Both of these proteins were found to occur in greater concentration in the sera of the old animals (Table V). Therefore, it is not possible to attribute the increased inhibiting action of the protein to either one of these proteins by itself. It is more probably due to their joint action. Even the quantity of euglobulin precipitated by CO_2 is greater in the sera of the older animals (Table V), indicating the probability that the euglobulin itself is not the stimulating substance, but rather some substance carried down with it. No rigid conclusion should be drawn here, however, since it is possible that this precipitate might contain some of the fibrin of the plasma from which the serum was made.

There is considerable variation among the sera of the individual animals of the same age in the concentration of the different protein fractions. Duplicate determinations on any one serum agreed closely, however, showing that this phenomenon is due to actual differences in the animals, and not to the experimental technique. The same variation is noted in the growth of fibroblasts in the sera of animals of the same age. Therefore, although the average results are obtained from figures varying to quite an extent, they indicate without any doubt greater inhibiting action in the serum protein of the older animals, and also a higher concentration of protein in the sera as the age of the animals increases. It seems probable that this inhibiting action is not due to any one protein, but rather to the protein as a whole.

Concentration of the Total Lipoid, Lecithin, and Cholesterol in Sera of Old and Young Animals.

An examination of the changes taking place in the lipoid of the serum with increasing age is even more important than that of the protein, since the lipoid is chiefly responsible for the inhibiting action. The total lipoid was determined in the sera of chickens 6 months old and 4 to 5 years old, the lecithin for 3 month, 6 month, and 4 to 5 year old chickens, and cholesterol for 3 month and 4 to 5 year old chickens. The total lipoid was determined as follows:

5 cc. of serum were poured drop by drop into 75 cc. of a mixture of alcohol and ether (3:1). The solution was heated to boiling, cooled, made up to 100 cc.,

[7] This fractionation was made by the use of sodium sulfate.

and filtered. An aliquot portion of the filtrate (75 cc.) was taken for analysis. Three times its volume of absolute ether was added. The precipitate obtained had no inhibiting action on fibroblasts, while the lipoids remaining in solution showed the usual retarding action. After centrifugation, a large aliquot (250 cc.) was evaporated to dryness in a tared flask, and the residue weighed. To ascertain whether the residue contained salts that would invalidate the results, the residue was redissolved, transferred to a platinum crucible, and ashed. A negligible amount of ash remained (0.0014 gm.), showing that the salts had been satisfactorily removed. The lipoid itself would, of course, yield a small quantity of ash. Therefore, no error from this source needs to be considered. In developing the above procedure, it seemed desirable to use a method resembling as nearly as possible that employed in preparing the lipoid fraction for the culture medium, especially since the usual methods of determining total lipoid are not very precise. Sufficiently large quantities of serum were used (5 cc.) to diminish the experimental error to less than 1 per cent.

TABLE VI.

Concentration of Total Lipoid in Sera of 6 Month Old and 4 to 5 Year Old Chickens.

6 mos. old		4 to 5 yrs. old	
Serum No.	Weight per 100 cc. gm.	Serum No.	Weight per 100 cc. gm.
2755	0.800	2754	1.133
2774	0.815	2773	1.055
333C	0.992	332C	0.993
2835	0.895	2834	1.042
		2848	1.072
Average.....	0.876		1.059

Lecithin in the sera was determined at first by the method of Randles and Knudson,[8] and later by Whitehorn's[9] modification of that method. The Whitehorn modification gave more uniform results than the technique of Randles and Knudson on duplicate determinations. The method of Bloor[10] was found to be the most suitable for determining cholesterol. As other experimenters have found, the colors did not always match the standard. Both a cholesterol standard and the inorganic standard of copper sulfate and sodium dichromate of

[8] Randles, F. S., and Knudson, A., *J. Biol. Chem.*, 1922, liii, 53.

[9] Whitehorn, J. C., *J. Biol. Chem.*, 1924, lxii, 133.

[10] Bloor, W. R., *J. Biol. Chem.*, 1916, xxiv, 227.

McMaster[11] were used. While these figures involve quite a large experimental error, there was no difficulty in distinguishing between

TABLE VII.

Concentration of Lecithin Phosphorus in Sera of Chickens of Different Ages.

3 mos. old			6 mos. old			4 to 5 yrs. old		
Serum No.	Mg. phosphorus per 100 cc. serum	Average	Serum No.	Mg. phosphorus per 100 cc. serum	Average	Serum No.	Mg. phosphorus per 100 cc. serum	Average
2871	4.80 5.40	5.10	2817	5.90	5.90	2754	6.70 6.50	6.60
2870	5.90 4.95	5.43	2333	5.00 5.10	5.05	2773	7.20 7.50	7.35
2859	4.50 4.60	4.55	2774	6.40 6.30	6.35	332C	6.90 7.50	7.20
2849	3.45 4.85	4.15	2755	6.10 5.40 5.20	5.57	2818	6.70 6.70	6.70
2835	4.80 4.60	4.70				2834	7.80 7.50	7.65
						2848	7.50 7.60	7.55
						2857	9.30	9.30
						2869	7.30 6.70	7.00
						2868	7.40 7.30	7.35
Average.....		4.78			5.72			7.41

the quantity of cholesterol present in the sera of the old and young chickens.

The results (Tables VI to VIII) show that there is an increase in

[11] McMaster, P. D., *J. Exp. Med.*, 1924, **xl**, 25.

TABLE VIII.

Concentration of Cholesterol in Sera from 3 Month and 4 to 5 Year Old Chickens.

3 mos. old			4 to 5 yrs. old		
Serum No.	Mg. cholesterol per 100 cc. serum	Average	Serum No.	Mg. cholesterol per 100 cc. serum	Average
2870	282 226 338	282	2869	166 188 200	185
2871	200 165 100	155	2868	83.0 95.3 95.3	91
2891	200 198	199	2889	75 68	71
2892	273 250	261	2904	187 220 250	219
2892	273 238	255	2905	107 107 99	104
2859	157 144 144	148	2857	170 200 200	190
2907	340 340 322	334	2754	192 205 196	198
2906	191 178 166	178	2818	161 134 128	141
2870	215 201 213	210	2834	87 80 86	84
Average.....		225			143

the total lipoid and also in the quantity of lecithin as the age of the chicken increases, but a decrease in the cholesterol.[12]

The total lipoid in the sera of the 4 to 5 year old chickens was 1.06 per cent and in the 6 month old, 0.876 per cent, giving a ratio of 1.21. The concentration of phosphorus due to lecithin was 4.78 mg. per 100 cc. in the sera of the 3 month old chickens, 5.72 mg. per 100 cc. in the sera of the 6 month old chickens, and 7.41 mg. per 100 cc. in the sera of the 4 to 5 year old chickens. The ratio of the 4 to 5 year old to the 6 month old animals was 1.29. These figures are in general accord with the results on growth in the serum lipoids of animals of these ages, and indicate that the inhibiting action is due, at least partly, to the lecithin which increases in concentration in old age. The fact that the concentration of cholesterol decreases as age increases, 225 mg. per 100 cc. at 3 months to 143 mg. per 100 cc. at 4 to 5 years, shows the necessity of further investigation in this field. The differences with age are so small, in comparison with the individual variations between animals of the same age, that it is impossible as yet to draw any conclusions concerning the part cholesterol plays in the increased inhibiting action of serum lipoids with age. As yet, the separate lecithin and cholesterol fractions of the sera have not been investigated in relation to their action on growing fibroblasts. A study of the action of pure cholesterol is now being undertaken.

DISCUSSION.

It is evident from the foregoing experiments that the changes in serum accompanying an increase in age, which render it more inhibiting to the growth of fibroblasts, occur in both the lipoids and the proteins of the serum. Although the serum protein is less inhibiting than the serum lipoid, the rate at which their retarding actions augment with age is approximately the same and also the same as the rate at which the retarding action of the serum itself augments with age. The change taking place in the protein fraction is due to the disappearance of a small quantity of growth-stimulating substance and also

[12] Roffo found an increase in both lecithin and cholesterol in rats between the ages of 3 and 5 months, but he made no examination of the sera of older rats. Roffo, A. H., *Compt. rend. Acad.*, 1925, clxxx, 1529.

an increase in the quantity of inhibiting protein. The concentration of each protein fraction of the serum is also greater as age advances. The increased inhibiting action of the serum lipoid in old age is associated with a higher concentration of total lipoid and of lecithin, and a smaller content of cholesterol than is present in the sera of young animals. An investigation of the action on fibroblasts of the lecithin and cholesterol fractions of the lipoid and of pure lecithin and cholesterol is necessary.

It would be interesting to know the mechanism by which these chemical substances act upon the cells and restrain their multiplication. Under normal conditions, the humors of the animal acquire a delicate balance between the growth-stimulating substances and those retarding growth, an understanding of which might give some information concerning abnormal conditions in which this balance is not maintained.

Probably there is also a delicate balance between conditions producing growth and those under which functioning of cells takes place. It is known that heart tissue kept in a medium unsuitable for growth will continue its muscular functioning, but this stops when nutritive substances are added to the tissue and growth takes place. In connection with this, it is interesting to note that the lipoids retard growth of tissues, but that an isolated heart perfused continuously with Ringer solution will cease beating, due to the loss of some substance removed by the Ringer solution, but will have that function restored on the addition of a small amount of the ether extract of serum.[13] Lecithin produces the same effect. As yet these phenomena are far from being understood.

The data at hand are also too meager to venture on any explanation of how lipoid and protein inhibit growth. However, since it has been discovered[14] that fibroblasts proliferate rapidly in proteose solutions and peptic digests of proteins, and that they live and multiply in the protein of embryo juice, it seems probable that this protein is first hydrolyzed to the proteose stage by means of enzymes either in the

[13] Clark, A. J., *J. Physiol.*, 1913–14, xlvii, 66.

[14] Carrel, A., and Baker, L. E., *J. Exp. Med.*, 1926, xliv, 503. Carrel, A., *Compt. rend. Soc. biol.*, 1926, xciv, 1060. Carrel, A., and Baker, L. E., *Compt. rend. Soc. biol.*, 1926, xcv, 359.

embryo juice or within the cells. It is known that serum exerts an antienzymatic action. It is possible, therefore, that the inhibiting action of serum on growth may be associated with its antienzymatic action, which would retard the production of nutritive hydrolytic products.

SUMMARY.

The increased inhibiting action of serum with age on the growth of fibroblasts has been shown to be due to changes in both the lipoids and the proteins.

Although the serum protein is less inhibiting than the serum lipoid, the rate at which their inhibiting action augments with age is approximately the same.

The change in the serum protein with age is due to the disappearance of a small quantity of growth-stimulating substance, and also to an increase in concentration of inhibiting protein. The concentration of all protein fractions becomes larger as age advances.[15]

The greater inhibiting action of the lipoid is associated with a higher concentration of total lipoid and lecithin, and a smaller content of cholesterol as the animal grows older.

The hypothesis is suggested that the inhibiting action of the serum is associated with its antienzymatic action.

[15] It is well known that the protein of serum increases as age advances. Investigators differ in their findings on the relations between age and the different protein fractions. Wells, C. E., *J. Biol. Chem.*, 1913, xv, 37. Hatai, S., *J. Biol. Chem.*, 1918, xxxv, 527. Toyama, I., *J. Biol. Chem.*, 1919, xxxviii, 161.

THE BACTERICIDAL PROPERTY OF COW'S MILK.

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Many have commented on the bactericidal properties of raw milk. It is well known that when certain bacteria are introduced into fresh raw milk their numbers decline for a time and subsequently multiply. Hesse[1] noted that *B. typhosus* and the cholera vibrio were prevented from multiplying in raw milk. Park[2] observed that the number of bacteria decreased in milk kept for 24 hours at 42°F., the same sample stored at 50°F. revealed a moderate increase in bacteria, and when the holding temperature reached that of the room or the incubator the bacterial increase was marked. Heinemann[3] found that milk contains for certain species of organisms a bactericidal substance, but the growth of other organisms is not inhibited. He further pointed out that the substance was destroyed at the boiling point or by heating to 60°C. for 30 minutes.

Rosenau[4] studied the phenomenon in considerable detail. He concluded that there is a diminution of the organisms in fresh raw milk and compared the action to that of a weak disinfectant and showed that the number of bacteria decreased markedly during the first 8 or 10 hours of incubation but rose rapidly thereafter. The action was prolonged but less rapid at 15°C. He attributes the apparent decrease largely to agglutination and calls attention to the possibility of phagocytosis by the leucocytes as an additional explanation for the decline.

More recently Chambers[5] showed that milk contains a definite bactericidal property which is destroyed by heating at 80° or 90°C. for 2 minutes. He found that the action was specific and depended on both the cow and the species of bacteria employed. He failed to find a relationship between agglutination and growth inhibition except that they were both destroyed by heat. It was pointed out that the lactic acid types of organisms were not inhibited in milk.

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[1] Hesse, W., *Z. Hyg. u. Infectionskrankh.*, 1894, xvii, 238.

[2] Park, W. H., *N. Y. Univ. Bull. Med. Sc.*, 1901, i, 71.

[3] Heinemann, P. G., The kinds of bacteria concerned in the souring of milk, Chicago, 1903.

[4] Rosenau, M. J., and McCoy, G. W., *U. S. Pub. Health and Marine Hosp. Service, Bull. 41*, 1908, 449.

[5] Chambers, W. H., *J. Bact.*, 1920, v, 527.

Sherman and Curran[6] inoculated fresh milk with young cultures of *Streptococcus lacticus*. There was lag in the fresh milk cultures for 30 minutes, but in the control tubes in which sterile milk was employed no lag occurred.

Hanssen[7] found that fresh raw milk would inhibit the growth of *B. typhosus* and *B. parathyphosus* B for 1 to 4 hours at 37°C. When the inoculated milk was kept at the temperature of the room the decrease was slower but more prolonged. He further showed that milk from single cows varied greatly in the inhibiting substance at different periods. This he attributes to variations in the ration, since he infers that the substance is due to the concentration of oxidizing enzymes in the milk. The enzymes supposedly originate in the food and their concentration in the milk is dependent on their concentration in the ration. He also showed that milk heated to 63°C. for 30 minutes and to 70°C. for 15 minutes still retained its inhibitory activity. 75°C. for 15 minutes inactivated the substance. This fact indicated to him that the substance in the milk was not derived from the alexin of the blood.

The phenomenon of bacterial decrease has been explained in several ways: lack of adaptation of the organisms employed (Stocking[8]), bacterial lag, agglutination-phagocytosis, and finally a definite property of fresh raw milk. A number of workers have failed to observe the phenomenon of growth inhibition in fresh raw milk.

If we should assume that milk possesses a definite bactericidal substance, perhaps its true purpose has been overlooked. Many have commented on its function, especially in regard to the keeping qualities of milk. That such is not its true function seems obvious. It is clear that the udder contains a relatively limited flora of organisms which have adapted themselves to their environment. Among the organisms usually encountered are streptococci and various types of micrococci; both are frequently associated with mastitis. It is also probable that many bacteria capable of multiplying in milk, such as the intestinal or vaginal flora, frequently come in contact with the ends of the teats; yet relatively rarely are organisms of this class encountered in the normal udder. When they occur they are usually associated with udder inflammation. Again, we are confronted with the problem of resistance to udder infection. Certain cows remain in the herd for several years without developing udder disease while

[6] Sherman, J. M., and Curran, H. R., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 15.

[7] Hanssen, F. S., *Brit. J. Exp. Path.*, 1924, v, 271.

[8] Stocking, W. A., *Rep. Conn. Agric. Exp. Station*, Storrs, 1904, 89.

others suffer from repeated attacks. As far as known, the blood and milk of the resistant group contain no more agglutinins than the same fluids of the susceptible animals. That the bactericidal substance in milk might be of considerable assistance in preventing the growth of organisms within the udder seemed worthy of investigation, particularly if it would throw light on the problem of resistance to mastitis. With these points in view a number of observations were made.

EXPERIMENTAL.

Methods.—Since our problem concerned itself with the possible influence of the inhibitory substance within the udder, we decided to test the milk against the non-hemolytic mastitis streptococcus, the organism we have found most frequently in udder infection. Our Culture 55 was isolated in 1917 and since that time has been carried on horse blood agar or plain agar slants. It is usually transferred four or five times a year. Presumably it has adapted itself to growth on artificial media but readily multiplies in milk. It has the further advantage of agglutinating slowly even with high titered agglutinin.

Milk was always drawn directly into sterile bottles and all precautions were taken to avoid contamination. Generally the samples were taken from the middle portions of the milking. The milk after chilling was rapidly centrifuged and a portion of the liquid between the cream line and sediment withdrawn. In this way a relatively fat-free milk containing very few cells and a limited number of organisms was obtained. The milk was then distributed in amounts of 1 cc. in sterile agglutination tubes containing a glass bead. A sample of boiled or autoclaved milk was also distributed in the same way for control purposes.

All tubes were then inoculated with one loop of an 18 hour broth culture of the mastitis streptococcus, which was diluted 100 times with broth. Each tube was shaken before incubation and at half-hourly intervals throughout the experiment. The contents of the tubes were withdrawn at various times and plated with 12 cc. of 2 per cent agar prepared from veal infusion. The number of colonies was counted after 24 hours incubation at 38°C.

Our methods largely eliminated the cells present in the milk as well as decreased the number of udder organisms. The repeated shaking of the tubes containing the glass beads prevented agglutination, since clumps could not be detected on microscopic examination.

When blood serum was used it was handled in a similar manner. It was always freshly obtained and relatively free from cells.

Experiment 1.—In the herd to which we have had access, we had the opportunity of testing the bactericidal action of the milk from four groups of cows. The first group consisted of native cows which had been in the herd for several years and had no history of mastitis. The second group had been injected on four occasions with killed cultures of the mastitis streptococcus. They had been recently im-

TABLE I.

The Bactericidal Activity of the Milk and Serum of 8 Cows for the Non-Hemolytic Mastitis Streptococcus.

Cow No.	Group	Milk + culture of mastitis streptococcus				Blood serum + culture of mastitis streptococcus				
		Number of colonies				Number of colonies				
		At once	After 2 hrs.	After 4 hrs.	After 8 hrs.	At once	After 2 hrs.	After 4 hrs.	After 8 hrs.	After 20 hrs.
1	Resistant	8,448	6,464	6,784	6,400	8,704	7,040	6,336	4,864	2,688
2	"	8,960	8,320	6,528	6,218	8,448	8,768	6,786	2,592	42
Sterilized milk	Control	9,344	24,768	Innumerable	Innumerable					
3	Vaccinated	4,928	4,540	3,520	20,100	5,120	5,824	4,480	4,350	Innumerable
4	"	4,740	3,710	3,392	22,000	4,992	5,760	5,120	2,560	41,600
Sterilized milk	Control	5,370	9,792	Innumerable	Innumerable					
5	Young cows in first	4,480	3,520	3,584	28,800	4,480	3,456	4,160	3,648	Innumerable
6	lactation period	4,480	3,390	4,800	Innumerable	4,160	3,748	3,712	4,544	"
Sterilized milk	Control	4,860	9,090	Innumerable	"					
7	Cows susceptible	4,416	3,588	3,584	"	4,416	4,864	4,544	5,376	"
8	to mastitis	4,544	3,904	3,392	"	4,288	4,991	3,776	2,240	"
Sterilized milk	Control	4,352	10,760	Innumerable	"					

ported from Tennessee and their udders were free from non-hemolytic streptococci when introduced into the herd. The third group consisted of young native animals in their first lactation period. The fourth series were animals which had had several attacks of mastitis. The findings were about the same for all the animals in each group so that only the details of two tests are given. The details of the results of the bactericidal activity of both the milk and blood serum are given in Table I. In these observations sterilized milk from the laboratory supply was used for control purposes.

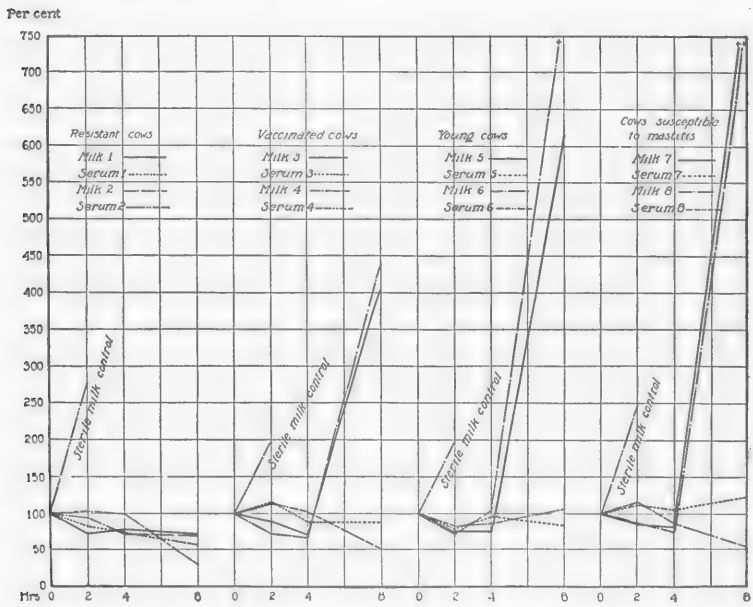

CHART 1. The inhibition of the mastitis streptococcus in milk and blood serum of resistant, vaccinated, and young cows and of cows susceptible to mastitis.

The data given in Table I have been plotted for the purpose of comparison and are shown in Chart 1. In the charts the number of colonies counted in the samples of both the raw and the boiled milk in each series have been added and an average struck; this represents 100 per cent, and departures from this line are recorded in terms of per cent. The results with the serum are given in the same way.

Both the table and the chart illustrate certain features. The milk

from all groups reacts in a similar manner. There is a sharp decline in the number of organisms during the first 4 hours of incubation, which is common to all groups. After this time there is a rapid multiplication particularly in the instance of the young cows and those susceptible to mastitis. The vaccinated group are about midway between the most resistant animals and the young and susceptible cows. The inhibition of the milk of the resistant cows is marked, since there is no multiplication during the first 8 hours. In sharp contrast is the rapid increase in the number of streptococci in the sterilized milk control. Here the number of organisms nearly doubled during the first 2 hours.

TABLE II.

The Bactericidal Activity of the Milk of a Resistant Cow and of a Young Cow Early in Its First Lactation Period.

	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Milk of resistant Cow 1	5,200	4,392	4,928	4,608	12,608
Boiled milk of Cow 1	5,000	48,900	Innumerable	Innumerable	Innumerable
Milk of young Cow 9, early in first lactation period	5,356	4,492	4,262	6,220	22,464
Boiled milk of Cow 9	5,068	36,990	Innumerable	Innumerable	Innumerable

It will be noted that the serum was in all cases inhibitory during 8 hours. This in itself is of little significance since the organism has not been trained to grow in serum and hence the lag period is relatively long. However, when the incubation period was increased to 20 hours, it was found that the serum of resistant Cows 1 and 2 was distinctly bactericidal and that of Cow 4 had definite inhibitory properties.

Inasmuch as in these observations both the milk and the blood were obtained in the morning and tested during the day, no opportunity was afforded for observations in the 6th hour, perhaps a critical point. This point has been covered in Experiment 2.

Experiment 2.—The milk of resistant Cow 1 and a young cow early in the first lactation period was obtained and employed in the manner outlined. In this and

ensuing observations the control consisted of a portion of the milk boiled for 5 minutes. The data have been tabulated in Table II.

From Table II it is clear that the inhibitory substance may be about as active in the milk of a young cow early in its first lactation period as that manifested in the secretion of an old cow which has passed through several lactation periods.

The behavior of the streptococcus in boiled milk affords a sharper contrast than the use of the stock milk medium which has been autoclaved. The former apparently represents an ideal medium, since it is the fresh product boiled for 5 minutes. The stock medium is prepared from commercial skim milk from the same dairy; it is older when separated and requires titration. It is of course heated to a higher temperature for a longer period.

Thus far it has been shown that the bactericidal substance is present to a varying degree in the milk of all cows examined. In addition to these reported, the milk from ten others has been examined with practically identical results. There is always a period from 4 to 8 hours in which the streptococcus fails to multiply. We are inclined to the opinion that during this period there is actual diminution succeeded by a period of multiplication. The streptococcus has thus far failed to coagulate the raw milk before the 8th hour, and in samples possessing strong inhibitory activity there is no coagulation at the end of 24 hours. From a number of observations we are convinced that most of the inhibitory substance is utilized during the first 8 hours, since as soon as the streptococcus begins to increase it continues to do so at a rapid rate. This is well borne out in Table I. Both the boiled milk and the sterilized milk controls are always definitely thickened at 8 hours and often firmly coagulated.

From Table I it appears as if there was a parallel between the activity of the blood serum and that of the milk; it seemed likely that the substance originated in the blood and appeared in a diluted form in the udder. One or two points, however, indicate that this explanation is not entirely true. If the substance originated in the blood, it might be possible to increase its concentration in the milk by immunization. The injection of dead cultures of streptococci into the cows of Group 2 (Table I) did not increase the concentration in the udder

above the usual level. The milk of young cows early in their first period of lactation compared in its inhibitory effect with that from the

TABLE III.

The Inhibitory Effect of Milk on Culture 55 and on a Freshly Isolated Strain of Mastitis Streptococci.

	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Milk of resistant Cow 1 +Culture 55	6,336	4,800	5,120	6,720	9,185
Boiled milk of Cow 1 + Culture 55	7,260	20,160	Innumerable	Innumerable	Innumerable
Milk of Cow 1 + freshly isolated streptococci	7,168	5,788	6,912	5,760	41,520
Boiled milk of Cow 1 + freshly isolated streptococci	7,296	16,588	Innumerable	Innumerable	Innumerable
Milk of young Cow 10, early in first lactation period + Culture 55	5,356	4,492	4,262	6,220	22,464
Boiled milk of Cow 10 +Culture 55	5,068	36,992	Innumerable	Innumerable	Innumerable
Raw milk of Cow 10 + freshly isolated streptococci	1,344	1,241	1,088	11,980	19,120
Boiled milk of Cow 10 + freshly isolated streptococci	1,197	30,538	Innumerable	Innumerable	Innumerable

others. It is to be presumed that these cows had been exposed to infection for too short a period to acquire resistance to the mastitis streptococcus.

Since we were convinced that the raw milk of all the cows contained a definite substance which inhibited the growth of the streptococcus, we decided to continue the study with the hope of throwing some light on its properties and its probable source. In Experiment 3 we attempted to show the influence of the culture on the inhibitory substance. The inhibition against Culture 55, previously referred to, and a freshly isolated strain of the mastitis streptococcus in the second culture generation were compared. The results are recorded in Table III.

It is apparent that the inhibitory substance in the milk of Cow 1 is a little more effective against the culture which has been carried on artificial media for a longer period than against the freshly isolated strain, although in the latter instance there is well marked inhibition. In the case of Cow 10, a young animal early in the first lactation period, the effect of the addition of the freshly isolated streptococcus is more pronounced, for inhibition occurred only within the first 4 hours. The experiment also indicates that the freshly isolated strains are better adapted for growth in the raw milk than the older strain isolated several years ago and since cultivated on artificial media.

Although the growth-inhibiting effect of whole or skim milk has been commented on by many, we have failed to find any reference whether the agent is contained in the whey. To test the effect of whey a number of experiments were made. Experiment 4 affords an example.

Experiment 4.—Commercial rennet tablets were ground and dissolved in sterile salt solution in the proportion of 2 tablets to 20 cc. of salt solution. The solution was sterilized by filtration through a Berkefeld candle N. 2.5 cc. of the filtrate was added to 50 cc. of fresh milk largely freed from fat, and after mixing was placed in a water bath at 38°C. Within 10 or 15 minutes sufficient whey was obtained for the tests. As a control procedure 2.5 cc. of salt solution was added to 50 cc. of the milk and the mixture heated in the water bath at 38°C. A third portion of the milk was boiled for 5 minutes. The whey, raw milk, and boiled milk were distributed in the usual manner and inoculated with the culture. The inhibitory effect of the whey and of the milk is given in Table IV.

It is apparent that whey contains the inhibitory substance to about the same extent as the milk. It cannot be said that the substance is greatly concentrated by the removal of the casein.

It has been shown by one of us[9] that milk will react to a relatively uniform level with a specific cow serum precipitin. The milk of Cow 1 used in this experiment reacted with cow serum precipitin at a maximum dilution of 1:640. The whey reacted at a dilution of 1:2,560. Therefore, in the process of rennet coagulation there is a considerable concentration of blood protein in whey. If the inhibitory agent comes from the blood then it is to be expected that it would be considerably concentrated within the whey. The protocols submitted in Table IV show that this is not the case.

Chambers demonstrated that 80° or 90°C. for 2 minutes would destroy the bactericidal effect of milk. Heinemann stated that

TABLE IV.

A Comparison of the Inhibitory Effect of Raw Milk and of Whey on the Mastitis Streptococcus.

	Streptococci present					
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.	After 20 hrs.
Milk of Cow 1	5,200	4,392	4,928	4,608	12,608	Innumerable
Whey of Cow 1	5,440	4,224	4,608	4,032	8,896	"
Milk of Cow 11	5,440	4,160	5,632	5,760	28,800	"
Whey of Cow 11	5,696	5,440	4,352	8,064	28,224	"
Combined milk of both cows, boiled for 5 min.	5,000	48,960	Innumerable	Innumerable	Innumerable	"

60°C. for 30 minutes would destroy the substance. Rosenau claimed that the inhibitory effect for *B. lactis aerogenes* was weakened at 55°C. and almost destroyed at 60°C. On the other hand, 60°C. did not affect the inhibition against typhoid. Hanssen states that the inhibitory substance for *B. typhosus* resists a temperature of 63°C. for 30 minutes and 70°C. for 15 minutes, although it is inactivated at 75°C. for 15 minutes. Since there is some difference of opinion in regard to the temperature required to inactivate or destroy the substance in milk, it was desirable to try the effect of various temperatures. For purposes of comparison we also observed the effect of

[9] Jones, F. S., *J. Exp. Med.*, 1926, xliii, 451.

the same temperatures on a cow blood serum of considerable bactericidal activity. It also seemed to us that if it was possible to show that the substance in milk was more or less resistant to heat than that of the blood, it would be regarded as additional evidence that they were different substances. That this really proved to be the case is brought out in Experiment 5.

Experiment 5.—Milk from Cow 13 which would inhibit growth of the mastitis streptococcus during the first 8 hours of incubation was obtained as usual. It was distributed in tubes in amounts of 10 cc. The contents of one tube was used in its raw state, another tube was boiled for 5 minutes, and the others were heated for 20 minutes at temperatures of 56°, 58°, 60°, 62°, 65°, 70°, and 80°C. The milk was then cooled and distributed in amounts of 1 cc. in the small tubes containing glass beads and inoculated with the culture. Cow 1 was bled in the afternoon and the blood stored in the refrigerator overnight. The serum was clear and free from cells. It was distributed in amounts of 7 cc. One tube was not heated. The others were heated for 20 minutes in the water bath at temperatures of 56°, 58°, 60°, 62°, and 65°C., and then all were inoculated with the culture. In this experiment we used a strain of the streptococcus which had been rapidly passed through broth which contained 50 per cent cow serum heated at 60°C. for 30 minutes. The culture was of the same age as the one used to inoculate the milk. The data are given in Table V.

It is apparent that the bactericidal factor for the mastitis streptococcus in the blood serum is inactivated at 56°C. With the increase in temperature inhibition of the substance is more marked. The serum heated at temperatures from 56° to 62°C. reveals no gross changes, but at 65°C. it becomes slightly opalescent; above 65°C. it coagulates. In contrast is the higher temperature required to inactivate the inhibitory agent in milk; temperatures ranging from 56° to 62°C. fail to affect it. When the temperature was increased to 65° or 70°C. for 20 minutes there was some impairment, although milk so treated proved inhibitory during the first 6 hours. 80°C. for 20 minutes is as efficient in inactivating the substance as boiling for 5 minutes.

The difference in the temperature required to inactivate the bactericidal substance in blood serum and in milk strongly suggests that the substances are different.

It is well known that antibody and blood proteins pass from the

TABLE V.

The Effect of Various Temperatures on the Bactericidal Activity of Milk and Cow Serum.

	Milk					Blood serum					
	Number of colonies					Number of colonies					
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.	After 20 hrs.
Unheated	4,352	5,584	3,008	2,304	3,456	8,000	6,592	7,168	5,248	5,056	6,720
Heated 20 min. at 56°C.	4,736	3,328	3,136	2,560	3,136	7,424	8,064	31,100	52,000	Innumerable	Innumerable
58°C.	4,480	3,200	2,560	3,136	3,392	8,064	9,600	48,900	Innumerable	"	"
60°C.	4,160	3,776	2,624	3,072	3,264	7,616	13,760	86,000	"	"	"
62°C.	4,480	3,072	2,626	2,944	3,392	8,000	17,920	Innumerable	"	"	"
65°C.	4,608	3,072	2,368	2,880	115,200	7,268	40,300	"	"	"	"
70°C.	4,608	3,072	3,328	3,072	115,200						
80°C.	3,264	46,000	Innumerable	Innumerable							
Boiled 5 min.	4,352	57,600	"	"							

milk into the blood. Ehrlich and Wassermann[10] noted that the milk contained from 1/15 to 1/30 as much antitoxin as the blood serum. Osborne and Wakeman[11] by analysis obtained 0.2 gm. of lactoglobulin per liter of milk. Crowther and Raistrick[12] and Wells and Osborne[13] were unable to distinguish lactoglobulin from serum globulin by chemical or anaphylactic methods. Howe[14] has shown that the blood of 30 months old cows contains on an average 5.06 gm. of globulin per liter. If, then, the dilute products of blood serum are responsible for inhibition, a medium containing about 1/25 part of fresh blood serum should prove inhibitory. That such is not the case is brought out in the following experiment.

Experiment 6.—The following medium was devised as a substitute for milk. Casein, 2.5 gm.; N/20 NaOH, 40 cc.; 0.9 per cent NaCl solution, 60 cc.; lactose, 5 gm. Autoclaved for 15 minutes at 15 pounds pressure. The reaction was pH 7.4. To 25 cc. of the above 1 cc. of sterile fresh serum from Cow 1 was added. This was distributed into small tubes and inoculated with the streptococcus. As a control procedure the medium which contained no serum was also inoculated with the culture. Plates were prepared from both series at once and after 2, 4, 6, and 8 hours incubation. As far as we could determine, growth took place to about the same degree in the medium containing serum as in the one which did not. There was an increase during the first 2 hours and a steady, though not rapid, increase thereafter.

It is true that an amount of serum proportionate to the globulin content of normal milk is insufficient to inhibit the growth of the non-hemolytic streptococcus in a relatively poor synthetic medium.

Rosenau has commented on the relative specificity of the reaction in milk since one of his samples restrained the typhoid bacillus and staphylococcus but not Parathyphoid A and B. In the sense that Rosenau used the term "specific" it is probably true, since in our hands milk which would restrain the streptococcus had little effect on one strain of *B. coli* and only a moderate restraining influence on *B. bovisepticus.*

[10] Ehrlich, P., and Wassermann, A., *Z. Hyg. u. Infectionskrankh.*, 1894, xviii, 239.

[11] Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1918, xxxiii, 7.

[12] Crowther, C., and Raistrick, H., *Biochem. J.*, 1916, x, 434.

[13] Wells, H. G., and Osborne, T. B., *J. Infect. Dis.*, 1921, xxix, 200.

[14] Howe, P. E., *J. Biol. Chem.*, 1922, liii, 479.

Thus far there has been no evidence submitted to show whether the multiplication of one species of organism will absorb the inhibitory substance for another. With this in view the following experiment was devised.

Experiment 7.—Milk from Cow 13 was collected as usual and after centrifuging was divided into lots of 10 cc. Lot 1 was refrigerated at once; Lot 2 was heated to 62°C.; to each 10 cc. of Lot 3 the growth from 5 cc. of an 18 hour broth culture of the streptococcus was added; and Lot 4 was treated in the same way except that each 10 cc. received the growth from 7.5 cc. of broth culture of *B. bovisepticus*. Another portion of the milk was boiled for 5 minutes. The tube heated at 62°C. for 20 minutes and the tubes to which the cultures were added were incubated at 38°C. for 6 hours. The tubes inoculated with the cultures were then heated at

TABLE VI.

The Effect of Absorption with Streptococci and B. bovisepticus on the Inhibitory Substance in Milk.

	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Raw milk, control	2,752	2,688	2,432	2,304	3,264
Milk heated at 62°C.	2,752	3,008	2,688	2,120	2,814
Absorbed with Streptococcus 55	3,200	4,736	Innumerable	Innumerable	Innumerable
Absorbed with *B. bovisepticus*	2,816	3,264	14,848	"	"
Boiled milk control	2,752	29,952	Innumerable	"	"

62°C. for 20 minutes. Exposure at this temperature was sufficient to kill the organisms but not inactivate the inhibitory property. It was necessary to adjust the reaction to the original pH of the milk of Lots 3 and 4 with N/Na_2CO_3. The effect of the absorption and heat was then tested in the usual manner after the addition of the mastitis streptococcus. The results are seen in Table VI.

Under the conditions it is apparent that the growth of one species of organism will absorb the inhibitory principle for another. It was not possible to completely absorb the substance, it is true, but the fact that for this purpose the strain of *B. bovisepticus* was about as efficient as the streptococcus argues that the substance is not specific for only the streptococcus.

We supposed from the frequent reference by other workers to fresh milk that this bactericidal substance deteriorated rapidly, even when kept at favorable temperatures. On several occasions we tested milk that had been stored at 6°C. for 2 or 3 days and found the efficiency of the inhibitory element to be unimpaired. After 10 days we failed to find any difference. After refrigeration for 27 days the milk was still capable of inhibiting the growth of the streptococcus for 4 hours with slow growth from this time onward. Originally it would inhibit 8 hours. The sample held for a period of 2 months contained a large number of putrefactive organisms and could not be tested.

DISCUSSION.

It is apparent that there is present in the milk of all the cows examined a substance which will inhibit the growth of mastitis streptococci for a definite period. The concentration of the inhibitory substance varies in the secretion of different animals. It may be as concentrated in a young cow early in its first lactation period as in an older, more resistant animal which has been repeatedly exposed to infection with streptococci. It is equally true that artificial immunization with killed cultures did not lead to an appreciable increase of the inhibiting agent in the milk. The inference is that the substance is a natural one since it is not increased by exposure to streptococcic infection or artificial immunization and because it is not specific for a single organism. The fact that the milk of young cows early in the first lactation period contains the substance in about the same amount as the older animals argues that it is inherent.

Whether or not such a substance under the present conditions of dairying may be regarded as of much value in protecting the udder against the rapid multiplication of bacteria which have gained entrance is open to question. However, under the most favorable conditions it is possible that a few organisms which have recently gained access to the udder might be prevented from multiplying until they are flushed out at the next milking. It may be that the substance is more potent than we suspect, since many opportunities are available for the entrance of bacteria into the teat canal although in the main the flora of the udder is limited to relatively few species and these

species have become well adapted to the environment and the inhibitory influence of this substance.

Under more natural conditions the substance might have considerable influence in protecting the udder. From the experiments the inhibition of growth of the mastitis streptococcus during the first 4, 6, or 8 hours was strong. Under natural conditions the calf would empty the udder at intervals which correspond to these periods. The inhibiting agent would act to prevent bacterial multiplication and thus insure milk of low bacterial content for the calf. The lengthening of the period between milking to 12 hours under the usual conditions of dairying has more or less rendered the substance inoperative.

Although we have no data directly bearing on the origin of the substance, by inference it seems possible to localize its source. Rosenau's contention that the diminution is due to agglutination is probably true for the organisms he tested. Our technique seems to rule out agglutinin, especially in view of the microorganism employed. The blood contains only weak agglutinin for the streptococcus, which is not easily agglutinated, and clumps could not be detected on microscopic examination of the milk.

If the substance is of blood origin the inhibitory substance should be greatly increased in the whey since with rennet coagulation there is a considerable concentration of the blood proteins, as shown by serum precipitin tests of the whey.

The view that the substance is "alexin" from the blood is not supported by our observations. Thus, in the normal udder there is only a slight interchange of blood proteins from the circulation to the milk and the concentration is too low to be a serious factor. This fact is borne out by the experiment in which fresh blood serum was added in the proportion of 1:25 to a special medium containing casein and lactose without inhibiting the growth of the streptococcus. The alexin from the blood of cows, like that of most mammals, is inactivated at a temperature of 56°C. for 20 minutes, while milk containing the inhibitory substance may be heated for the same period at 62°C. without impairing its inhibitory properties. It is not completely inactivated at 65° and 70°C. In this respect, then, there is a distinct difference between the substance in the milk and that of the blood. We infer from these facts that the substance originates in the udder.

It however resembles alexin in that it deteriorates slowly even at a temperature of 6°C.

Hanssen's contention that the substance is an oxidizing enzyme must be given consideration. He noted that the inhibition was more marked in the cow on pasture than in the milk of the same cow during the winter. From this he inferred that the substances originated in the food. The cows in our observations were fed about the same ration throughout the year.

Our experiments further indicate that the action of the substance is not specific, since by incubating raw milk with one organism we were able to absorb the inhibiting agent for another.

SUMMARY.

The bactericidal activity of fresh raw milk from a number of cows has been tested with the non-hemolytic mastitis streptococcus. By using this organism and other means we were able to rule out the action of agglutinin. The milk of all cows examined inhibited the growth of the streptococcus for definite periods. The length of the inhibition period varied; the milk from some cows prevented growth for 8 hours, that of others for only 4 or 6 hours. The inhibitory action may be as strong in the milk of a young cow in its first lactation period as in that of an old cow known to be resistant to udder infection. It is possible to absorb the streptococcus inhibitory substance by first inoculating the milk with *B. bovisepticus*. We were unable to show that the substance was increased by artificial immunization of cows with the streptococcus.

Whey obtained by the action of sterile rennet solution inhibited the growth of the streptococcus to about the same extent as the milk from which it was obtained.

We infer that the substance originates in the udder since it differs from blood alexin in its resistance to heat, it is not increased in the whey although the blood proteins are more concentrated, and it is not increased in the milk when the cows are artificially immunized or repeatedly exposed to natural infection.

THE RELATION BETWEEN INVASION OF THE DIGESTIVE TRACT BY PARATYPHOID BACILLI AND DISEASE.

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The large group comprising the colon, paratyphoid, and typhoid bacilli have certain characters in common which have led most students of this group to assume a close genetic relationship. The production of disease by members of the colon group resides in a capacity to multiply in the lower small intestines with coincident production of a toxin or toxins. These are absorbed and produce certain effects leading to extreme congestion of the capillary system and hemorrhage especially marked in the kidneys. The capacity to produce disease among the highly parasitic types of the paratyphoid group, such as the hog cholera bacillus, consists in the power to penetrate the mucosa, lodge and multiply in certain viscera, chiefly spleen, lymphoid tissue of the intestines, and liver, where a toxin similar to that produced by *B. coli* comes into intimate contact with certain vulnerable cells. Between these extremes we may assume that there exist many varieties either approaching one or the other of these types or so balanced that multiplication in the intestinal tube and the invasion of the tissues and multiplication therein both contribute to the disease.

The extensive literature dealing with epidemics of food poisoning and with the clinical paratyphoid disease in man serves to trace the different types of disease. Some are evidently due chiefly to gastrointestinal intoxication; others to invasion of the body, in which respect they approximate the behavior of the typhoid bacillus. If the above grading of the colon-typhoid group is true, it follows that the invasion of the digestive tract in itself may or may not be followed by clinical manifestations. In the colon bacilli it would be necessary that they multiply abundantly in the tube itself in order to produce

enough toxin; for the other extreme, that they penetrate the mucosa and find no resistance to temporary multiplication in the blood or lymph channels or even within certain cells. The immediate bearing of the disinfecting action of the stomach and its relation to penetration of bacteria through the mucosa of mouth and throat instead of the intestinal wall need not enter into the problem, nor the obvious fact that different host species will react differently when invaded by the same type of bacteria. Moreover, certain abnormal states of the digestive tract may modify bacterial behavior.

The general question here raised has an important bearing upon an understanding of vaccinations against this group of bacteria. In the case of human typhoid the subcutaneous injection of bacilli killed by heat has the support of statistics as to its efficiency. The injection of dead bacteria tends to protect the body whose digestive tract is invaded by typhoid bacilli. Does the typhoid bacillus multiply in the lower small intestine after introduction or only later in the disease after it has been discharged through the bile and from local ulcers into a tube whose protective mechanism has been disorganized by the disease process? Are there races of typhoid, some capable of multiplying in the intestines at the start, others promptly invasive? The vaccine probably has no effect on bacilli in the tube but only after they have penetrated the mucosa. Disease might conceivably be produced by races simulating the colon type even after vaccinal protection.

In approaching questions of this kind experimentally, the nearer our experiment to natural conditions, as far as known, the nearer our results approach the solution of the natural or practical problem. At the same time any other combination of experimental procedures may assist in analyzing the problem and thus provide material for a future synthesis approaching practical conditions.

The experiments to be reported were begun in 1921 before the extensive researches on mouse typhoid by Flexner and Amoss, Topley, and Webster[1] had appeared, and were closed in August, 1923. They are incomplete but they furnish some data of value in the final

[1] For a good bibliography of this work up to 1926 see Lockhart, L. P., *J. Hyg.*, 1926, xxv, 50.

formulation of factors governing the rise and fall of mortality in this group of diseases.

Methods.

To bring the problem within the field of experiment, the hog cholera bacillus and the white mouse were chosen. The susceptibility of gray mice to this bacillus had been studied by one of us in 1885. At this time the breeding of white mice for experimental purposes had scarcely begun. The white and the gray mouse have about the same degree of susceptibility towards the hog cholera bacillus. The gray mouse, however, as usually obtained in traps is a more variable subject often showing lesions of the kidneys and other organs due to various parasites and possibly poisons. The strain of the hog cholera bacillus used in the following experiments had been under cultivation for about 6 months when the experiments were begun.[2]

It is obvious that this relationship of hog cholera to white mice is artificial and that an analysis of it can cover only a narrow portion of the entire field. The minimum fatal dose, given subcutaneously, varied considerably from case to case. An 18 to 24 hour bouillon culture in which the final clouding is fairly constant was used in all cases. In 1921 the surely fatal dose was about 0.05 cc. Nearly 50 per cent succumbed when 0.02 cc. was injected. A small number died from doses down to 0.002 cc. Death resulting from a minimum dose usually occurred on the 7th to 9th day, rarely later. Doses representing multiples of the minimum dose killed within 4 days. In later experiments (1922–23) the minimum fatal dose was evidently somewhat larger. In an animal as small as the mouse the skill with which the infecting dose is deposited in the subcutis in each mouse, not too deep into the muscular tissue or even the peritoneal cavity nor into the skin itself, probably determines in part the death rate. No attempt was made to increase by passages the virulence of the strain used. Each experiment was started with the stock culture grown on sloped agar and kept at about 38–40°F. in the refrigerator after each monthly transfer had been incubated 1 or 2 days.

EXPERIMENTAL.

From the 50 or more mice used in preliminary trials which received subcutaneous doses of hog cholera bacilli and survived, the injected bacilli were demonstrated in the spleens in all cases up to the 75th day, the longest period tested. The spleens, usually larger than in the normal condition, were torn into three or four pieces which were placed in tubes of sloped agar. There was evident a gradual diminution in the number of colonies as the period between inoculation and

[2] This strain was kindly sent by Dr. Dimock of the University of Kentucky.

killing widened. In mice killed when sick in the early days up to the 10th the colonies were numerous. In those active and apparently well when chloroformed they were scarce even in bits of spleen as large as split peas.

After considerable experimentation with one lot of white mice, a

TABLE I.

Mouse No.	Subcutaneous dose	Died (d.) or killed (k.)	Cultures (spleen)	Remarks
	cc.			
1	0.05	d. 7 days	Many colonies	
2	0.05	d. 9 "	" "	
3	0.01	k. 25 "	Few "	Some pneumonic foci
4	0.01	k. 25 "	" "	" " "
5	0.01	d. 9 "	Many "	
6	0.01	d. 10 "	" "	

TABLE II.

Mouse No.	Dose fed	Died (d.) or killed (k.)	Spleen cultures
	cc.		
1	6	d. 11 days	Many colonies
2	6	k. 25 "	Few "
3	6	d. 13 "	100 "
4	6	k. 15 "	Growth only in condensation water
5	6	d. 7 "	Many colonies
6	6	k. 26 "	1 colony
7	6	d. 11 "	Many colonies
8	6	k. 15 "	" "

pneumonia appeared which entered as a disturbing factor as Table I indicates.

It will be noted that of the four mice receiving 0.01 cc. subcutaneously the two affected with pneumonia survived. Another lot of mice was raised from a small group obtained from an isolated colony and this remained free from the pneumonia.

Inasmuch as the problem before us concerned the relation of the digestive tract to infection, feeding of cultures was resorted to.

An 18 to 24 hour bouillon culture was used in a definite amount to moisten a small piece of dog biscuit which was placed in a solid watch-glass in the container, a battery jar with wire mesh cover. The mice were of the nearly uniform age of 2 months when used. They were all of the new lot which was free from mouse typhoid and pneumonia. The food consisted of corn, oats, and dog biscuit, with a constant supply of water. Before infecting the mice they were fasted overnight.

TABLE III.

New Stock of Mice.

Mouse No.	Bouillon culture fed	Died (d.) or killed (k.)	Spleen cultures	Remarks
	cc.			
47	0.2	d. 7 days	Many colonies	
48	0.5	d. 7½ "	" "	Cysticerci in liver*
49	0.5	d. 6 "	" "	" " "
50	1.0	d. 7 "	" "	
51	2.0	k. 9 "	" "	" " "
52	3.0	d. 7 "	" "	Very sick when killed
		Old Stock.		
59	0.2	k. 28 days	+−−**	
60	0.5	d. 10 "	Many colonies	
61	0.5	k. 28 "	+++	
62	1.0	d. 9 "	Many colonies	
63	2.0	k. 34 "	+++	
64	3.0	k. 34 "	++−	

* Only the original stock of new mice were infested. The offspring were free.
** 3 pieces of spleen in 3 tubes of agar. 2 remained sterile.

As a rule the food placed before them was entirely consumed within 18 to 24 hours. In the earlier experiments two mice were fed in a jar and kept together. The irregular results pointed to the possibility that one of the two mice had eaten the bulk of the infected food. This surmise is borne out by the experiment shown in Table II.

It will be seen from the table that only one of every pair died. The other must have obtained some of the food, for spleen cultures were positive 15 to 26 days after the feeding when the surviving, still

active mouse of every pair was chloroformed. Thereafter only one mouse was fed in a jar.

Before giving up the old stock of mice a test was made of their resistance to the hog cholera bacillus as compared with that of the new stock. Six mice each were fed as described and each kept by itself in a jar.

It will be noted that five out of six of the new stock died. The sixth was very sick when chloroformed and would have probably died. The tapeworm infection of the original mice of the new stock, as observed in other mice of this lot, did not influence the outcome. Of the old stock only two out of six died. The remaining four were killed after 28 to 34 days. It will also be observed that the result did not go parallel with the dose fed.

TABLE IV.

Mouse No.	Dose fed	Died (d.) or killed (k.)	Spleen cultures
	cc.		
17	7	d. 6 days	Many colonies
18	Control	k. 25 "	Sterile
19	7	k. 19 "	A few colonies
20	Control	k. 25 "	Sterile
21	7	d. 7 "	20 colonies
22	Control	k. 25 "	Sterile
23	7	d. 6 "	Many colonies
24	Control	k. 31 "	Sterile

As a preliminary test the degree of infectivity of mice fed with hog cholera bacilli to other mice associated with them was tried in the following way.

Mice were distributed one in a jar and, after withholding food, fed with dog bread moistened with a fresh bouillon culture. A very large dose was given the mice purposely to bring about as heavy a discharge of bacilli as possible. On the following day the fed mice were removed to clean jars and a fresh mouse added to each jar. All but one of the fed mice died, with the usual postmortem appearances and positive spleen cultures. The one survivor was killed on the 19th day. The spleen culture was positive. The exposed mice remained active and were killed 25 to 31 days after the beginning of the exposure. The spleen cultures made with large bits of tissue remained sterile.

In a second test, after feeding, the fed and the control mice were placed together in a wire cage. They were killed in pairs after 4, 5, 6, and 8 days respectively. In none of the four exposed mice were hog cholera bacilli detected, in the spleen, abdominal cavity, duodenum, ileum, and feces. Thus far the experiments indicated in all cases an invasion of the body after feeding cultures as shown by the presence of hog cholera bacilli in the spleen in all survivors for an indefinite period up to $2\frac{1}{2}$ months. Contacts with mice fed large doses did not carry bacilli in their spleens or digestive tracts.

It was next decided to analyze in some detail the condition of the digestive tract following feeding.

To ensure uniformity the mice used were from the new stock bred originally from only a small number of individuals. They were all about 2 months old and weighed from 17 to 23 gm. The diet and method of feeding remained the same. The culture was fed on a small piece of dog biscuit.

Cultures were made from certain regions of the alimentary tract and in some experiments the material was weighed after suspension in a definite amount of bouillon and a calibrated loop of this rubbed over agar plates in order that some rough conception of the numbers of hog cholera bacilli present might be gained. Cultures from the intestinal contents required careful manipulation since hog cholera bacilli were present in the abdominal cavity of most, if not all, infected mice. The portion of intestine desired was cut out and removed to a sterile petri dish. With sterile instruments the tube was cut longitudinally. In most cases the walls curled outward, exposing the contents, so that they could be removed with forceps without difficulty. The contents were suspended in bouillon. Feces were removed by holding the living mouse as if for injection and gently rubbing the abdomen with the finger. When feces were ejected, they were taken with sterile forceps and put into bouillon and shaken. From these shaken suspensions of intestinal contents and feces, plates were made directly with one loopful for each plate. The suspension was then kept at room temperature overnight. If the hog cholera organism did not appear on the first set of plates, the next day duplicate ones with malachite green were made from the incubated material. Two agar plates were made from each specimen, one contained 0.3 cc. of a 1 per cent aqueous solution of malachite green per 10 cc. of agar. The plain agar plate made possible a general survey of the intestinal flora of each mouse, and the malachite green agar inhibited growth of nearly all but the hog cholera bacillus. Occasionally there was found on the plates an organism which grew like the hog cholera bacillus. A large bubble of gas in lactose broth and negative agglutination tests with a specific rabbit serum sufficed to distinguish this organism from the hog cholera bacillus.

The first series of tests to be described determined roughly the number of organisms found in the stomach after the beginning of feeding and the survival time. The 18 to 24 hour bouillon culture

TABLE V.

Bacteria in Stomach of Fed Mice.

Mouse No.	Killed after feeding	Bacteria in 0.15 gm. stomach contents	Calculated total in stomach contents	Stomach contents actually plated
		First Test		
	hrs.	*millions*	*millions*	*gm.*
113	2	65	261	0.00005
114	3	195	716	0.00005
115	4	15	37	0.00005
116	5	68	157	0.00005
117	26	0	0	0.00005
118	29	0	0	0.00005
119	48	0	0	0.0005
		Second Test		
120	2	233	570	
121	3	74	294	
122	5	83	168	
123	6	77	116	
124	8	231	231	
125	18	16	28	
126	24	0	0	Incubated contents also negative
127	51	0	0	
		Third Test		
		thousands	*thousands*	
168	18	24	151	0.0009
169	20	91	427	0.0009
170	22	<1	<1	0.0009
171	24	0	0	Incubated contents also negative

was added in amounts of 0.5 cc. to a gm. of dog biscuit and each mouse fed separately in a mouse jar. Three separate tests were made. In the two first the mice were left in the jars in which they

had been fed. In the third they were removed to clean jars and fresh food given after 17 hours.

The results agree in demonstrating the presence of large numbers of hog cholera bacilli during the early hours after feeding. A decline occurs about the 18th hour and in 24 hours the fed bacilli are no longer detected in the contents.

Hydrogen ion determinations of stomach contents, according to Brown's drop method,[3] were made on three mice killed 1¼ hours after a meal of corn and oats. The pH readings were 5.4, 5.6, and 5.8.

In a second series of experiments comprising three lots of mice, 10, 10, and 12 in number, respectively, an attempt was made to follow the fed bacteria beyond the stomach into the lower digestive tract, the peritoneal cavity, and the spleen.

In the first series the mice were fed each 0.5 cc. of bouillon added to 3 gm. of dog biscuit. The 1st mouse was killed 24 hours after the beginning of the meal, and one on each succeeding day, except the 5th day. The 10th mouse was therefore killed on the 11th day after feeding. Spleen cultures were positive in all but the 1st mouse. Cultures from the peritoneal cavity were positive in all but the 2nd and 4th mouse. The fed bacilli were obtained on plain and malachite green agar plates from contents of the ileum in all cases; in the 4th and 7th mouse, however, only after incubating the contents in bouillon overnight. On the plain agar plates the fed bacilli were overgrown by colon bacilli in the 3rd, 6th, and 9th mouse.

In the second and the third series the examination was extended to duodenal contents and feces and to the 21st day after feeding.

The results agree with those of the first series. Two mice died in the third series after 9 and 11 days. One mouse in the second series was negative throughout. In addition to these series, two mice were fed and kept 28 days. The spleen cultures were positive. Plates from duodenum, cecum, and feces contained colonies of hog cholera bacilli in both mice. A few bacilli were found in contents of ileum of one only.

The prompt invasion of the peritoneal cavity and spleen by way of the digestive tract suggested a final experiment in which the reverse

[3] Brown, J. H., *J. Lab. and Clin. Med.*, 1923–24, ix, 239.

route was to be traced. Three small groups of mice received subcutaneously 0.02 cc. of a 24 hour bouillon culture in 0.1 cc. fluid. They were chloroformed up to 41 days after injection and the contents of ileum, cecum, colon, and discharged feces spread on plain and malachite green agar plates.

TABLE VI, *a*.

Killed, days after feeding	Cultures			No. of organisms per 0.016 gm. of ileum contents
	Spleen	Duodenum	Feces	
1	−	−	+	11,600
2	+	−	−	2,227
3	+	+	+	6,032
4	+	−	+	39,771
6	−	−	−	0
7	+	+	+	18,560
8	+	+	+	185,600
9	+	+	+	659,569
10	+	−	+	2,735
11	+	−	−	0***

TABLE VI, *b*.

11	+	+	+	5,290
12	+	+	+	3,648
13	+	−	+	214
14	+	−	+	4,690
15	+	+	+	21,458
16	+	−	−	0
18	+	−	+	0***
19	+	−	+	426
20	+	−	+	95
21	+	+	+	0***

*** Recovered on plates after incubating material in bouillon overnight.

Among the first group of eight, one died within 24 hours of some undetermined cause. Invasion of the intestinal tract was evident after 3, 7, and 15 days. Mice killed after 2, 5, and 21 days respectively were negative in this series. In the second group of eleven mice, five died. These mice were smaller than those used hitherto. All

but one of the six survivors were sick when killed. In all the intestinal tract contained hog cholera bacilli. The third group of

TABLE VII.

Killed, days after injection	Cultures				Condition of mouse when killed
	Ileum	Cecum	Large intestine	Feces	
			First Group		
2	−	−	−	−	Well
3	+	−	−	−	"
5	−	−	−	−	Sick
7	+	+	+	−	"
8	−	−	−	+	Well
15	+	+	+	+	"
21	−	−	−	−	"
			Second Group		
7	+	+	+	+	Sick
8	+	+	+	+	Very sick
9	+	+	+	−	" "
10	+	+	+	+	" "
13	+	+	+	+	" "
16	+	+	+	+	Well
			Third Group		
12	−	−	−	−	Well
13	+	−	−	−	Sick
14	+	+	−	−	Slightly ill
16	+	+	+	+	Well
17	+	−	+	+	"
19	−	−	+	−	?
21	+	+	+	+	Well
37	−	−	+	−	"
39	−	−	−	−	"
41	+	−	+	+	"

twelve mice was more resistant. Only two died in 4 and 9 days, respectively. In two mice killed after 12 and 39 days respectively injected bacteria were not recovered.

A control experiment was made to eliminate the chance that the injected culture dilution might ooze out locally, be lapped by the mouse, and introduced into the digestive tract directly. During the injection the needle was inserted under the skin about 1 cm. The skin was subsequently washed with 95 per cent alcohol, dried, collodion applied to the punctured area and allowed to dry before the mouse was caged. Six mice received 0.02 cc. culture fluid in 0.1 cc. bouillon dilution. Two died after 6 and 7 days, respectively. The rest were killed after 7, 8, 9, and 12 days. All had the injected bacteria in contents of cecum, colon, and in the feces. In all but one (9th day) bacteria were found also in the ileum.

DISCUSSION.

Before we attempt an interpretation of the experimental work, a brief statement of the lesions following the feeding or injection of hog cholera bacilli in mice may be helpful. Following a minimum fatal dose injected subcutaneously mice die in 7 to 9 days, rarely later. If the mouse is chloroformed when manifestly sick, the mucosa of the intestinal tract will be found with nearly intact epithelium. The gross lesions observed involve liver and spleen. Examination of fixed and hardened tissue show involvement of lymph nodes, mesenteries, and peritoneum generally and to a certain degree of the mucosa of the large and more rarely of the small intestine.

The lesions of the liver are the most constant and characteristic. Hog cholera bacilli just isolated from swine produce sharply defined necroses, 1 to 2 mm. in diameter.[4] These rarely appear following inoculation with strains under prolonged cultivation. The lesions always encountered comprise the presence of cells of endothelial type in the sinusoids. The entire liver is involved in this condition. The sinusoids may contain a few cells not encroaching on the liver tissue, or many closely packed, tubercle-like, and causing disappearance of surrounding liver cells. The spleen is always large, dark red, firm. The tissue changes differ according to the severity of the induced disease. In the fatal cases, there is much hemorrhage or at least marked distention of the pulp vessels and the focal accumulation of endothelial cells. In recovering cases the lymph follicles are large. The mesenteries are broadened by cell accumulations of endothelial and lymphoid type. The lymph nodes are enlarged and the changes like those going on in the spleen.

[4] Boxmeyer, C. H., *J. Med. Research*, 1903, ix, 146.

The intestines interest us more particularly in view of the special problem of this communication. In the presumably fatal cases there may be found here and there in the sections of the large intestine accumulations of endothelial cells in the mucosa, forcing the crypts apart or else causing them to disappear. The infiltrated zones are as a rule still covered with epithelium. Actual ulcers were very rare in our cases. The large intestine is the dominant seat of such infiltrations, although they may also be found in the small intestine. There is no evidence that Peyer's patches or lymphoid follicles are involved to any degree. They are not the necessary loci for the changes. The feeding of large doses does not lead to diffuse injury to the surface epithelium or the free portions of the villi such as might be looked for when a toxin is freely discharged from multiplying bacteria and such as we note when poisons have been ingested. Nor do we observe the severe congestions often leading to hemorrhages which are associated with unrestricted multiplication of *B. coli* in the small intestine of calves.

The hog cholera organism is evidently of a more highly adapted, parasitic type than the cultures used by Webster and Topley. The disease follows subcutaneous inoculation and intraperitoneal injections are unnecessary. Deaths earlier than 4 days are rare. They tend to occur between the 7th and 9th day more or less independently of the dose within limits. These facts indicate a greater penetration and resistance to destruction and a more stabilized march of the disease process. Acute intoxication and early death is produced only by many multiples of the surely fatal dose.

The main outcome of these studies is the demonstration of the readiness with which hog cholera bacilli pass into the blood and lymph circulation after ingestion in food and the uniformity with which they make their appearance in the intestinal tract after subcutaneous injection in surviving mice. The second point to be noted is the persistence of the fed or injected bacilli in the spleen, even though in very small numbers. The prompt dissemination may be ascribed in part at least to the small size of the animal, the delicate tissues, and the short distances to be travelled. On the other hand, the universal invasion without marked illness or death suggests that, to compensate for this early entrance, the mouse is endowed with a high degree of

fundamental resistance, or natural immunity, which represses multiplication. This is evident when the mouse is compared with the rabbit which succumbs to the minimum lethal dose of the mouse.

Clinical disease manifested in the mouse by crouching on top of the bedding with staring coat and somnolent behavior is not followed by recovery. If the mouse is killed at this time, many bacteria are found in the spleen, and the obvious inference is that disease is associated with multiplication of the bacilli in the viscera and when this has reached a certain momentum reversal of the process and recovery are no longer possible. The relation of feeding to clinical disease and death is not cleared up by the experiments reported. As we trace the fed bacteria down, we note during the first hours large numbers in the stomach contents which completely disappear within 24 hours. Some have probably passed into the duodenum. The numbers found in the ileum may represent those passed into the small intestine from the stomach and they may be the residuum of a process of destruction and multiplication going on side by side. 2 weeks after feeding there are no bacilli found or else only a few in this section of the small intestine. Their discharge in the formed feces goes on parallel with their presence in the digestive tract and spleen. On the whole the assumption is warranted that clinical disease depends on the numbers that penetrate into the body proper and multiply there. The conditions favoring penetration are unknown. The passage of bacilli outward may be due in part to the causes operating in favoring penetration inward, but in view of the uniform and diffuse involvement of the liver it is more probable that they are discharged in the bile. Lesions of the intestinal tract characterized by ulceration of the mucosa are very rare and they may play a very subordinate part in the outward discharge.

If multiplication in and consequent intoxication from the alimentary tract were factors when large doses are fed, we should expect the appearance of symptoms within 2 days, allowing for mass destruction in the stomach during the 1st day. This does not occur. The evidence points to penetration and subsequent multiplication reaching a climax within a week. The marked irregularity in the effects of small and large doses of hog cholera bacilli fed indicates that the presence of such bacilli in the intestinal tract of mice does not imply a

corresponding absorption into the body proper, although such absorption takes place since bacilli are always detected in the spleen later on. A large dose fed may evidently be followed by slight absorption and if we assume that it is the numbers absorbed that determine the clinical manifestations, the marked absence of agreement between the doses fed and active disease is satisfactorily explained. The concept of absorption includes both penetration into the body and capacity to multiply. Here the individual immunity of the host must come into play. In these studies no attention has been paid to the possible relation of the minute nematodes of the cecum to the appearance of disease after feeding, nor were the intestinal contents examined for the overmultiplication of flagellates, occasionally observed in mice.

The passage of bacteria into the system from the digestive tract may stand in some causal relation to motility. To the writers' knowledge attention has not been called to this possible relationship. This function is remarkably persistent in the paratyphoid group under cultivation and is possessed by all pathogenic members of the group. In fact its persistence in bouillon cultures over 24 hours old, in the sense that it is shared by most organisms in the culture, is presumptive evidence that the organism belongs to the above group rather than to *B. coli*. Its persistence in a pathogenic group strongly argues for its usefulness and in the penetration of the normal mucosa it may explain the invasiveness of the hog cholera bacillus as compared with *B. coli*. After its entry into the system in small numbers by this method even in the presence of large numbers in the alimentary tract, a slight increase in resistance may suppress multiplication, whereas the introduction parenterally even of small doses may soon exhaust the normal, antibacterial forces. Experiments to test the significance of motility are under way.

CONCLUSIONS.

Hog cholera bacilli fed to mice disappear from the stomach within 24 hours, but remain and perhaps multiply in the ileum for at least several weeks. They promptly penetrate the mucosa and may be found in the spleen. Bacilli introduced subcutaneously quickly pass

into the intestinal tract where they may be found for some weeks. Infected mice may harbor bacilli in the spleen for several months.

Mice possess a relatively high degree of natural resistance towards hog cholera bacilli which gives way to large doses. Disease is probably the result of the invasion of the viscera from the digestive tract following feeding, but the relation between the dose fed and the numbers penetrating the mucosa is a variable one and the conditions favoring such invasion not determined. Contact with mice discharging bacilli failed to cause recognizable invasion of the digestive tract or the viscera.

STUDIES ON A PARATYPHOID INFECTION IN GUINEA PIGS.

I. Report of a Natural Outbreak of Paratyphoid in a Guinea Pig Population.

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During the summer of 1924 a spontaneous outbreak of paratyphoid occurred among the guinea pigs maintained for breeding purposes and for stock. Throughout a 5 year period prior to the outbreak the guinea pig population had been conspicuously free from disease. No animals had been introduced from outside sources during this time. The stock represented a rather homogeneous population but not a vigorously inbred one. Inasmuch as the outbreak was of spontaneous origin in a uniform population known to have been free from disease for a period of years, it was deemed worthy of study. The infection was followed through the active stage of the epidemic* and an endemic stage which subsequently ensued. Particular attention was paid to the latter stage in an endeavor to ascertain some of the factors accountable for the maintenance of the infection in the form of sporadic cases and carriers. The present paper will deal only with the course of the outbreak, the gross pathology of the disease, and the bacteriology of the causal organism. The factors which seemed significant in determining the maintenance of the infection through the endemic period will be discussed in a second paper.

Numerous references to spontaneous paratyphoid in the guinea pig may be found in the literature. The disease appears to have been recognized as early as 1884 and subsequently called bacillary pseudotuberculosis of the guinea pig

* We are indebted to Dr. Laura Florence for making autopsies and cultures of guinea pigs during July and August of 1924.

by Eberth (1) in 1885. T. Smith and J. R. Stewart (2) in 1897 reported a single case of pseudotuberculosis in a guinea pig and were able to isolate an organism from the spleen which was culturally related to the hog cholera bacillus and to *B. typhi murium* of Loeffler. Wherry (3) in 1908, investigating the efficacy of the "Azoa" virus for the extermination of rats, encountered the disease in his guinea pig stock. He isolated a bacterium apparently identical with that found by Smith and Stewart. He named it *B. choleræ caviæ* but later changed it to *B. pestis caviæ*. On the basis of direct agglutination he judged it to be identical with the "Azoa" bacterium. It showed only remote relationship to a human strain of *B. enteritidis*. Howell and Schultz (4) in 1922 reported a serious outbreak of guinea pig paratyphoid with high mortality. They isolated a number of different strains of *B. paratyphi*. All of the strains were distinct from *B. paratyphi* a and b, *B. enteritidis*, and *B. suipestifer*. Thomas (5) in 1924 encountered spontaneous infection in a small series of experimental guinea pigs. By agglutination tests he identified the organism isolated from fatal cases as either *B. enteritidis* or *B. paratyphi* of animal origin. He described the gross and microscopic pathology of the disease. A detailed summary of the literature pertaining to guinea pig paratyphoid up to 1916 is given by Holman (6) in a survey of spontaneous infections of the guinea pig. Paratyphoid has been studied in the mouse from the standpoint of experimental epidemiology, and reference should be made to the extensive researches of Webster (7), Topley (8), and their associates.

The guinea pigs comprising the population in which the outbreaks of paratyphoid occurred are maintained in three groups. The first group includes the breeding animals, divided into units of 5 sows and 1 boar kept in single cages. At parturition each sow with her young is removed to a separate cage and kept there until the young guinea pigs are weaned, a period of about 2 weeks. This forms the second group, which is maintained in the same room with the breeding stock proper. The cages are of metal, well separated, and offer only a remote possibility of direct communication. As soon as the young guinea pigs are weaned the sow is returned to the original breeding unit, the young separated as to sex and added to the stock benches. Here are segregated young pigs of the same sex and approximately the same age in groups of 20 to 30 individuals. They are the weaned, stock guinea pigs for general laboratory use and constitute the third group. They are maintained in a separate room from the breeders and unweaned young. During the months of July and August, in order to stop breeding approximately half of the boars are removed from the breeding units to individual cages in a separate room. Such

replacements as become necessary among the breeders are made from young guinea pigs selected from the stock.

The epidemic began on the 14th of July, 1924, and continued for a period of 8 weeks. At that time the population was composed of 597 animals distributed as follows: breeders 310, unweaned young 69, stock pigs 218. During the 8 week interval 104 individuals succumbed to the infection. Irrespective of groups the mortality rate for the period was approximately 17 per 100 of population. The distribution of deaths within the population was of some interest. The infection first appeared among the breeders which numbered, at the time, 250 sows and 60 boars. As previously stated, half of the boars had been removed from the breeding units during the summer, so there were actually only 30 males in the breeding room. During the 1st week 6 sows died from paratyphoid. The number of deaths

TABLE I.

Deaths per Week among Breeders during the Epidemic Period.

July 14–20	July 21–27	July 28–Aug. 3	Aug. 4–10	Aug. 11–17	Aug. 18–24	Aug. 25–31	Sept. 1–7
6	8	2	10	8	4	2	3

per week increased slightly, to 10 animals, during the 4th week and then declined. A total of 43 individuals from this group succumbed to the infection; all of them were sows. No deaths occurred among the boars exposed to infection in the breeding units nor among those isolated in individual cages. The number of deaths per week among the breeders during the 8 week period is given in Table I.

Exactly a week after the epidemic began the first deaths occurred among the stock pigs which numbered, at the time, 218 individuals. The number of deaths per week reached a peak during the 3rd week and then declined. There was a total of 50 deaths for the 8 week period. The number of deaths per week is given in Table II.

The appearance of the infection among the guinea pigs of the second group, sows with unweaned young, practically coincided with its appearance among the stock pigs. During the 8 week period a total of 11 deaths occurred among the 69 individuals which composed the

population of unweaned young at the start. Data are incomplete as to the number of sows with young during the 8 week interval. 1 sow succumbed to the infection. The number of deaths per week is given in Table III.

The active stage of the epidemic lasted 8 weeks and was followed by an endemic period, characterized by sporadic deaths, which has continued to the present time (May, 1926). During the year 1925, a total of 229 deaths from all causes occurred in the guinea pig population. Of that total, 91 animals showed sufficient evidence to warrant the diagnosis of paratyphoid as the cause of death. Those guinea pigs that showed no evidence of specific infection were generally

TABLE II.

Deaths per Week among Stock Guinea Pigs during the Epidemic Period.

July 14–20	July 21–27	July 28–Aug. 3	Aug. 4–10	Aug. 11–17	Aug. 18–24	Aug. 25–31	Sept. 1–7
0	11	16	6	8	8	0	1

TABLE III.

Deaths per Week among Unweaned Young during the Epidemic Period.

July 14–20	July 21–27	July 28–Aug. 3	Aug. 4–10	Aug. 11–17	Aug. 18–24	Aug. 25–31	Sept. 1–7
0	2	0	1	3	5	0	0

young, unweaned individuals that died shortly after birth. In most cases they appeared to be individuals that had received insufficient nourishment from the dam. The total of non-specific deaths included, in addition, a smaller number of stock guinea pigs and breeders that showed a variety of abnormal conditions upon autopsy.

The distribution of specific deaths within the population during the year 1925 was as follows: breeders, 23; stock guinea pigs, 28; unweaned young, 40. Distributed as to sex there were 23 deaths among males and 62 deaths among females. Data were lacking in a few instances. The total number of deaths per month, the number of deaths from paratyphoid, the percentage mortality from paratyphoid, and the estimated population by months are given in Table IV.

The gross pathological manifestations of the disease, encountered at autopsy, varied considerably. The most constant lesion, the one which led to the early name of pseudotuberculosis, was found in the spleen. In typical cases the surface was studded with foci varying in size from minute white points, barely discernible, to large yellowish nodules projecting above the surface and measuring several millimeters in diameter. Focal changes were found in the depths of the splenic tissue as well as on the surface. In addition there was marked enlargement and congestion of the spleen. With more acute cases death

TABLE IV.

Total Deaths, Deaths from Paratyphoid, Percentage Mortality from Paratyphoid, and Population by Months during 1925.

Month	Population	Total deaths	Deaths from paratyphoid	Percentage mortality from paratyphoid
January.....	856	8	3	0.35
February.....	1,105	12	3	0.27
March.....	1,006	9	3	0.29
April.....	800	25	17	2.15
May.....	677	14	5	0.73
June.....	524	44	21	4.00
July.....	401	51	10	2.49
August.....	460	8	5	1.08
September.....	438	27	16	3.65
October.....	500	7	5	1.00
November.....	540	9	3	0.55
December.....	581	15	0	0.00

resulted before focal changes had occurred and the spleen showed only enlargement and congestion. There were gradations between the two extremes marked by the presence of occasional foci. In several instances an exudative membrane partially covered the surface of the spleen.

The liver often showed foci varying in number and in size. At times there was marked congestion and at times fatty degeneration. In some instances the gall bladder was distended by a thin, purulent fluid. The intestinal tract, particularly the small intestine and cecum, showed congestion. In the cecum small, circumscribed areas of

hemorrhage were often present. There was generally an involvement of Peyer's patches, which in the more acute cases were swollen and showed small gray points giving a stippled appearance. In the less acute cases in which sufficient time had elapsed for the appearance of focal changes in other tissues, the same were generally discernible in Peyer's patches as distinct nodules, white or yellowish in color, and varying in number from one to sufficient to pack the entire patch. Lymphoid tissue in other localities, the mesenteric nodes and cervical nodes, often showed involvement varying from swelling with congestion to the presence, in addition, of foci. Less constant changes noted in other organs were an anemic condition of the kidney and a patchy congestion of the lung.

The genital tract often showed involvement. In the female there was congestion of the uterus with enlargement and accompanied sometimes by exudative plaques on the peritoneal surface and sometimes by the presence of a thin purulent exudate on cross-section. In the male changes were less frequent. At times the testis showed congestion and appeared swollen and soft. The seminal vesicles presented an opaque appearance with or without congestion.

As a routine procedure a culture was made from the spleen of all guinea pigs that came to autopsy. In every instance where the postmortem findings indicated paratyphoid there was obtained from the spleen a microorganism which showed cultural and biochemical characteristics typical of the paratyphoid members of the Salmonella group. Morphologically the microorganism was an actively motile, Gram-negative bacillus. On agar it produced colonies which were transparent, flat, of a bluish cast, non-mucoid, with regular border, and reaching a size up to 4–5 mm. in diameter after 48 hours on a thinly seeded plate. In broth a dense, even turbidity was produced in 24 hours with scant sediment and sometimes a partial surface film. Hydrogen sulfide was produced from peptone, but no indole. Gelatin was not liquefied. Of carbohydrates dextrose, maltose, mannitol, and dulcitol were fermented with acid and gas. There was no fermentation with saccharose, lactose, and salicin. In milk a transient acidity was produced with a slow change to an alkaline reaction. After 10 days to 2 weeks the milk presented a yellowish, semitranslucent appearance.

The bacteriological technic employed at postmortem was as follows.

With young guinea pigs the entire spleen was removed with sterile forceps to an agar slant containing a small amount of bouillon at the base. With adult animals one or several pieces of spleen were torn off and added to agar. In special cases cultures were made from other organs and from the feces. The tubes were examined after 18 hours and 48 hours of incubation at 37°C. If growth had occurred microscopic examination was made for motility. *B. paratyphi* shows active motility after 18 hours incubation, whereas most of the other bacteria, such as strains of *B. coli*, encountered at times in the spleen are non-motile or very sluggishly motile after that period of growth. Non-motile bacteria were not further identified. If the microorganism was motile additional preliminary tests were made in some cases. Generally when an 18 hours motility was encountered a pure culture of *B. paratyphi* was obtained. In doubtful cases where the 18 hours growth showed a few motile forms additional tests were always made. A transfer from the original culture was made to malachite green-lead acetate bouillon. If the tube showed a brown precipitate with motile bacteria after 18 hours of incubation, a lead acetate plate was streaked and several of the brown colonies which developed transferred to agar slants. In some instances the fermentation reaction was tried in saccharose and lactose bouillon.

In all cases the cultures tentatively identified as *B. paratyphi* were tested with an agglutinating serum. The antiserums were obtained from rabbits immunized with a culture isolated from a typical case of paratyphoid early in the endemic period. The first serum employed showed an agglutination limit in a dilution of 1:25,600 for its homologous culture. With it were tested a representative number of cultures isolated during the active period of the epidemic and all cultures isolated during the endemic period through August, 1925. The second serum showed an agglutination limit in a dilution of 1:51,200 and was used for testing all cultures isolated from August, 1925 to the present time. The method employed in the direct agglutination tests was as follows. The growth on an 18 hour agar slant of the organism was removed with saline and the suspension diluted to a turbidity corresponding to 2.4 on the Gates scale. 0.5 cc. amounts of the antiserum, ranging by twofold dilution from 1:100 to 1:25,600, were pipetted into small tubes and 0.5 cc. of the fresh unheated suspension added to each, giving a final dilution range of 1:200 to 1:51,200. Appropriate controls were included. The reaction was read after 3 hours incubation at 37°C. and again after 18 hours at ice box temperature.

Of 110 cultures tentatively identified as *B. paratyphi* which were tested by one or the other of the two serums only one failed to agglutinate to the titer limit of the serum for its homologous strain. The single exception was a culture isolated from a sow which died early in the endemic period with typical paratyphoid lesions. The culture

showed positive presumptive tests for *B. paratyphi* but was agglutinated only through a dilution of 1:3,200 by a serum with titer limit in a dilution of 1:25,600. A number of cultures were encountered which showed spontaneous flocculation in saline. In some instances cultures showed slight agglutination in a dilution higher than that of the previously determined titer limit of the serum.

TABLE V.

Agglutination Limits with Guinea Pig Paratyphoid 922 and Mouse Paratyphoid I Antiserums.

Culture	Antiserum	
	Guinea Pig 922	Mouse I
Guinea pig paratyphoid 922	1:51,200	1:51,200
" " " IV	1:200	1:200
" " " VII	1:51,200	1:51,200
" " " VIII	1:51,200	1:51,200
" " " IX	1:51,200	1:51,200
Rabbit " 933	1:51,200	
Mouse " I	1:51,200	1:51,200
" " II	1:200	1:200
Rat " V	1:51,200	
Swine " IV	1:400	1:400
Calf " I	1:400	1:400
" " IV	1:400	1:400
B. choleræ suis X	1:100	1:100
" *enteritidis* Gaertner (Kral)	1:400	1:400
" " " (Mt. Sinai Hospital)	1:800	
" *paratyphi* a Schottmüller	1:200	1:200
" " b "	1:400	1:400
" " *aertrycke* 387	1:200	1:200
" *typhi* X	1:3,200	1:1,600

In general the agglutination tests showed a marked uniformity of the cultures isolated. A single strain of *B. paratyphi* thus appears to have been the causal organism of the epidemic during both the active and the latent stages. The one culture which showed an outstanding difference in agglutinability may have been a different type of *B. paratyphi*. If so, it was apparently of no further significance as low agglutinating cultures were not subsequently encountered.

The relationship of the causal organism of the epidemic to other

members of the Salmonella group was studied by direct agglutination. The agglutination limits of 18 members of the Salmonella group and of one strain of *B. typhi* with an antiserum prepared against the epidemic strain are given in Table V. There are included, in addition, the agglutination limits with an antiserum prepared against a culture of Mouse Typhus I. This culture was originally isolated by Lynch (9) during a spontaneous epidemic of paratyphoid in mice. The two mouse strains were obtained from The Rockefeller Institute through Dr. Ida W. Pritchett. The *aertrycke* strain was obtained from Dr. E. O. Jordan. The rabbit strain was isolated from the stock of this Department in 1925. Of the guinea pig strains,

TABLE VI.

Agglutinin Absorption Tests with Guinea Pig and Mouse Typhus I Serums.

Serum	Culture	Direct agglutination	Absorbing culture	Absorbing dose	Agglutination after absorption	Culture
G.P.T. 922	G.P.T. 922	1:51,200	G.P.T. 922	1:5	1:50	G.P.T. 922
			" 922	1:5	1:50	M.T. I
	M.T. I	1:51,200	M.T. I	1:5	1:100	G.P.T. 922
			" I	1:5	1:100	M.T. I
M.T. I	" I	1:51,200	" I	1:5	1:50	" I
			" I	1:5	1:50	G.P.T. 922
	G.P.T. 922	1:51,200	G.P.T. 922	1:5	1:100	M.T. I
			" 922	1:5	1:100	G.P.T. 922

Culture 922 was isolated during the present epidemic. Culture IV was isolated by Dr. P. A. Lewis from a Boston epidemic in 1908. Culture VII was isolated by Dr. Theobald Smith in 1913, Culture VIII by Dr. Carl TenBroeck in 1914 from The Rockefeller Institute stock, and Culture IX from the same stock in 1917. The other cultures were selected from the collection maintained at the Department.

Reciprocal absorption tests, as a further indication of the relationship of the various cultures to the epidemic strain, were made only with Mouse Typhus I. The absorptive capacity of the mouse strain was compared with that of the guinea pig strain, Culture 922. The

method suggested by Krumwiede and his associates (10) was employed. The absorptive capacities of the two cultures in homologous and heterologous serums are given in Table VI.

DISCUSSION.

There was no indication that the active stage of the epidemic was preceded by a pre-epidemic period marked by occasional deaths. Routine examination of all guinea pigs that died among the stock had been made prior to the appearance of the early cases and nothing suggestive of the disease had been encountered. The infection began abruptly, ran a moderately acute course for a period of 8 weeks, and then subsided in part. The active stage was followed by a long protracted endemic period characterized by sporadic deaths. During both stages female guinea pigs showed a somewhat greater susceptibility to the infection than did the males. This difference in susceptibility was well brought out with the breeders during the active stage. Similar differences in susceptibility have been noted with other outbreaks of paratyphoid. With the adult animals, breeders, and older stock guinea pigs, the disease did not run a particularly acute course. At postmortem it was customary to find well developed lesions the nature of which would indicate that the disease had been in progress for about 1 to 2 weeks. With the younger guinea pigs, unweaned and recently weaned individuals, the disease was more acute. The unweaned animals sometimes died 2 to 3 days after birth with no noticeable manifestations save a slightly enlarged and congested spleen yielding *B. paratyphi* in pure culture. The cultures isolated at postmortem, with one exception, were of the same strain of *B. paratyphi*.

The comparative study of the agglutinative affinities of the epidemic strain was not extensive enough to permit definite conclusions as to its position in the Salmonella group. Direct agglutination indicated that the epidemic strain was closely related to three stock strains of guinea pig origin and to three strains from other rodents. It showed only remote relationship to a fourth guinea pig strain, to several types of human *B. paratyphi*, to *B. enteritidis* Gaertner, and to a group of strains isolated from domestic animals. Absorption tests with Mouse Paratyphoid I and with Guinea Pig Paratyphoid 922 gave

further proof of a close relationship between the two strains, if not of their actual identity. The indicated relationships are in harmony with the early work of Wherry (3) and the more recent work of Krumwiede, Valentine, and Kohn (11). The latter investigators studied the agglutinative affinities of fifteen strains from guinea pigs. Thirteen of the strains were alike and identical, as shown by agglutinin absorption, with two strains from mice and one each from a rabbit and a cat. They concluded that a distinct paratyphoid type or group of bacilli was encountered in spontaneous infections of laboratory animals especially rodents.

It is apparent that other members of the Salmonella group may be encountered, at times, in natural infections of the guinea pig and of other small animals as well, strains closely related to *B. enteritidis* Gaertner and but remotely related to the type discussed. Likewise, it is to be expected that the rodent strain will be found occasionally in larger animals exposed to infection through contact with rats and mice.

SUMMARY.

The course of a natural outbreak of paratyphoid in a normal guinea pig population is outlined. A description is given of the gross pathology of the disease. The bacteriology of the causal organism is discussed with particular reference to its agglutinative affinities.

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STUDIES ON A PARATYPHOID INFECTION IN GUINEA PIGS.

II. Factors Involved in the Transition from Epidemic to Endemic Phase.

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The active stage of the epidemic of guinea pig paratyphoid (1), occurring in the summer months of 1924, was followed by a long protracted endemic period studied up to June, 1926. During this period the percentage mortality showed considerable fluctuation from month to month. It was low when compared with the rate during the active stage except during the hot months of 1925 when there was a slight increase in the fatal cases. In the hope that a study of all cases of paratyphoid occurring subsequent to the early epidemic period might offer some explanation of the drop from the epidemic to the low endemic stage, attention was focused in particular upon the dams and their litters.

It has already been stated that during the epidemic period the mortality among the sows was high. During the later period and up to the present, June, 1926, only 4 sows died of the disease. During the endemic period all sows whose young showed infection with *B. paratyphi* were taken from the breeding house with the surviving young and placed together in a large screened box for observation. These sows and the remainder of the litter were killed at intervals and cultures made from spleen, uterus, gall bladder, and cecum. The blood serum was tested for specific agglutinins towards the epidemic strain. In all, 35 sows with at least one fatal case of paratyphoid in each litter were segregated during the period of observation.

On the basis of the postmortem findings the sows could be divided into three groups. The first group comprised the fatal cases. Death

TABLE I.

Bacteriological Data of Isolated Guinea Pigs and of Their Unweaned Young Which Had Succumbed to Paratyphoid Infection.

No. of guinea pig	Age at death in mos. k. = killed d. = died	Data of sow*						Data of young				
		Spleen	Uterus	Cecum	Gall bladder	Fetal spleen	Serum titer	No. in litter	Deaths	Date of death	Age in days	Spleen culture
										1924		
1	30 (k. Mar. 3, 1925)	+					1:320	3	1	Oct. 16	21	+
2	11 (k. Mar. 25, 1925)	−	−	−	−		1:160	3	1	" 24	6	+
3	25 (k. Mar. 25, 1925)	+	−	−	−		1:80	3	1	" 27	10	+
4	28 (k. Apr. 7, 1925)	−	+	−	−		1:160	4	1	Nov. 1	5	
										1925		
5	12 (k. Apr. 22, 1925)	−	−**	−	−	−	1:320	4	1	Jan. 24	7	+
6	10 (k. Mar. 19, 1925)	−	−**	−	−	1. + 2. − 3. −	1:80	4	1	Apr. 13	3	+
7	11 (k. Apr. 18, 1925)	−	−	−	−		1:80	4	1	" 13	3	+
8	15 (k. Apr. 21, 1925)	−	−	−	−		<1:10	4	1	" 16	3	+
9	25 (k. Apr. 18, 1925)	+	−	−	−		1:40	4	3	" 16	8	+
										" 16	8	+
										" 16	8	+
10	8 (d. Apr. 20, 1925)	+	+	+	+			5	3	Apr. 18		+
										" 18		+
										" 20		+
11	27 (k. Apr. 29, 1925)	−	−	−	−		1:160	2	1	" 25	10	+
12	12 (d. June 13, 1925)	+	+					4	2	June 12	8	+
										" 12	8	+
13	16 (k. Oct. 2, 1925)	−	−	−	−		1:40	3	2	Sept. 17	12	+
										" 17	12	+
14	17 (k. Oct. 2, 1925)	−	−	−	−		1:320	3	1	" 18	8	+
15	17 (k. Oct. 2, 1925)	−	−	−	−		1:20	4	1	" 19	11	+
16	16 (k. Oct. 13, 1925)	+	−**	−	−	1. − 2. −	1:80	4	3	" 29	21	+
										Oct. 8	30	+
										" 12	34	+

* + = positive cultures; − = negative cultures.

** Culture included placenta.

TABLE I—*Concluded.*

No. of guinea pig	Age at death in mos. k. = killed d. = died	Data of sow*						Data of young				
		Spleen	Uterus	Cecum	Gall bladder	Fetal spleen	Serum titer	No. in litter	Deaths	Date of death	Age in days	Spleen culture
										1925		
17	7 (k. Oct. 19, 1925)	−	−	−	−		1:1,280	2	2	Oct. 5	11	+
										" 5	11	+
										1926		
18	21 (d. Mar. 1, 1926)	+	+	+	+			5	4	Feb. 19	3	+
										" 19	3	+
										" 23	7	+
										" 23	7	+
19	20 (d. Feb. 27, 1926)	+	+	+	+			5	4	" 23	7	+
										" 25	9	+
										" 25	9	+
										" 25	10	+
20	32 (k. Mar. 8, 1926)	−	−	−	−		1:320	3	1	" 16	8	+

occurred some time after parturition. The sows all showed typical focal lesions in the spleen together with other manifestations of paratyphoid. *B. paratyphi* was obtained in cultures from the spleen and feces, and in two of the cases from the uterus and gall bladder. The blood serum, tested for agglutinins in only one instance, showed no agglutination in a dilution of 1:10. With these cases the majority of the young in the litter died.

The second group included the majority of surviving cases, *i.e.* sows which had recovered from a slight attack of paratyphoid or had been carriers with localization of *B. paratyphi* in the spleen. Within the litters only part of the young succumbed to paratyphoid. The sows of this group showed serum agglutinins for the epidemic strain of *B. paratyphi* in dilutions ranging from 1:20 to 1:1,280. The organism was recovered from the spleen in only two instances. Cultures from the feces and gall bladder were uniformly negative. In one case *B. paratyphi* was obtained from the uterus and in one case from a fetal spleen. It sometimes happened that the sow had been

rebred before removal for the nursing period. The spleen and liver sometimes showed a few foci, with a negative culture, together with other indications of past infection.

The sows of the third group showed no specific agglutinins in a dilution of 1:5. Cultures from the spleen, gall bladder, uterus, and feces were uniformly negative. The spleen and other organs did not show gross changes suggestive of former disease. The sows of the group were regarded as sometime fecal carriers of the organism. The postmortem findings of a selected number of the isolated sows, together with the young of their litters which succumbed to paratyphoid, are given in Table I.

This material affords a basis for certain inferences. (1) The dams, as might have been anticipated, were far more resistant than their offspring. The survivors appeared normal when killed later. (2) In those animals that died, the gross lesions appeared equal in extent and severity to those encountered in the epidemic period. (3) Intrauterine transmission of the infecting agent to the fetus was demonstrated in one case. (4) The bacilli were excreted either by way of the digestive tract, the uterus, or the mammary gland, which in one instance contained a number of abscesses. *B. paratyphi* was regularly isolated from the feces of those sows which succumbed to active infection shortly after parturition. With the sows which showed evidence of inactive infection or of recovery from a past active infection the causal organism was rarely recovered from the feces except when examination was made immediately after segregation. It seemed probable, however, that the majority of the sows were excreting the organism at the time of parturition. If such was the case, it would appear that the carrier state, as evidenced by the presence of *B. paratyphi* in the feces, was of relatively short duration.

To obtain indirect evidence bearing on the persistence of the causal organism in the feces a small series of guinea pigs was fed *B. paratyphi* and the feces subsequently cultured for the organism. The series included one lot of two older stock guinea pigs and one lot of four recently weaned pigs. For comparison a series of eight mice was included. Cultures made from feces collected before feeding were all negative. The animals were fed by pipette on 2 successive days. The guinea pigs received a total volume of 1 cc. of an 18 hour broth

culture of *B. paratyphi*, approximately 800,000,000 organisms. The mice received a total of 0.1 cc. of the same culture, or 80,000,000 organisms. Cultures were made 4 days after the last feeding and then every 7 days until negative. The mice invariably showed a long continued carrier state and were killed before the excretion of the organism had terminated.

The method used in culturing was as follows:

A heavy suspension of fresh feces was made in plain bouillon and incubated at 37°C. for 3 to 5 hours. A small amount of the culture, 0.2 cc., was pipetted into a tube of fermented bouillon containing lead acetate (0.25 cc. of a 1 per cent solution in 5 cc. of medium) and malachite green (0.1 cc. of a 0.1 per cent solution in 5 cc. of medium), and incubated overnight. In the presence of a deep brown or black precipitate and motility, or either, a drop was streaked on the surface of a lead acetate-fermented bouillon agar plate and a thin layer of the same medium superimposed. Deep brown colonies, after 18 hours of incubation, were fished to agar slants and finally tested with a specific antiserum. In the absence of *B. paratyphi*, the lead acetate-malachite green broth generally showed a very scant turbidity, no change in color of the precipitate, and no motility. In the presence of *B. paratyphi* there was always motility, a moderate turbidity, and a browning or blackening of the precipitate.

The guinea pigs were killed shortly after the indicated termination of the fecal carrier state. The mice were killed after a prolonged period of continued excretion. The results of the feeding experiments with the two lots of guinea pigs and with the mice are given in Tables II and III.

Per os administration of *B. paratyphi* to these guinea pigs was not followed by active disease. With the older stock pigs there was an immediate excretion of the organism which continued for a period of approximately 3 weeks. The feces subsequently became negative. The guinea pigs were then killed and autopsied. Lesions were not observed. The organism was recovered from a piece of Peyer's patch of the small intestine in one case and from the spleen in one case. Agglutination tests gave evidence of an immunity response on the part of the host. With the recently weaned guinea pigs there was a delayed and subsequently irregular excretion of *B. paratyphi*. One guinea pig gave but a single culture of the organism from the feces. The fecal excretion of *B. paratyphi* terminated sometime

TABLE II.

Results of Fecal and Postmortem Cultures from Guinea Pigs Fed B. paratyphi.

No. of days after last feeding	Stock guinea pigs		Recently weaned guinea pigs			
	1	2	3	4	5	6
4	Feces +	Feces +	Feces −	Feces −	Feces −	Feces −
11	" +	" +	" +	" −	" +	" +
18	" +	" +	" −	" −	" +	" +
25	" −	" −	" +	" +	" −	" −
27	" −	" −				
28	Killed Spleen + Peyer's patch***+ Serum agglutinins 1:320	Killed Spleen − Peyer's patch + Serum agglutinins 1:640				
32			" −	" −	" −	" −
34			" −	" −	" −	" −
35			Killed Spleen + Peyer's patch + Serum agglutinins 1:40	Killed Spleen + Peyer's patch − Serum agglutinins 1:80	Killed Spleen − Peyer's patch − Serum agglutinins 1:80	Killed Spleen + Peyer's patch − Serum agglutinins 1:20

*** "Peyer's patch" means that a piece of intestinal wall containing a Peyer's patch was cut out and transferred with some fecal material to an agar slant.

TABLE III.

Results of Fecal and Postmortem Cultures from Mice Fed B. paratyphi.

No. of days after last feeding	M 1	M 2	M 3	M 4	M 5	M 6	M 7	M 8
4	Feces +	Feces +	Feces +	Feces +	Feces +	Feces +	Feces +	Feces +
11	" +	Died on 10th day Spleen + Feces +	" +	" +	" +	" +	Died on 7th day Spleen + Feces +	" +
18	" +		" +	" +	" +	" +		" +
25	" +		" +	" +	" +	" +		" +
32	" −		" +	" +	" +	" +		" +
39	" +		" +	" +	" +	" +		" +
46	" +		" +	" +	" +	" +		" +
53	" +		" +	" +	Killed on 49th day Spleen + Feces +	Killed on 49th day Spleen + Feces +		Killed on 49th day Spleen + Feces +
56	Killed Spleen + Feces +		Killed Spleen + Feces +	Killed Spleen + Feces +				

between the 3rd and 4th week. At autopsy the organism was recovered from the intestine in one case and from the spleen in three out of four cases. The immunity response was weak as compared with that of the older guinea pigs.

With mice the feeding of the organism resulted in death in two cases. The survivors showed a persistent fecal excretion of *B. paratyphi*. The animals were killed at the end of the 7th or 8th week after the last feeding and in every case a positive culture was obtained from the spleen and from the feces.

During the endemic period, guinea pigs in other lines of research were scrutinized as far as this was possible A series of 32 stock guinea pigs had each received intraperitoneal injection of material containing *B. abortus*. At varying intervals after injection the animals were killed and the entire spleen cultured. In two cases pure cultures of the epidemic strain of *B. paratyphi* were obtained from the spleen. No gross manifestations of infection were noted in either case. Another series of 10 guinea pigs had received intraperitoneally sublethal doses of a virulent strain of *B. coli*. The animals were killed on the 5th day and the entire spleen cultured in every case. A mixed growth of the injected organism and the epidemic strain of *B. paratyphi* was obtained from one animal. The spleen showed only the exudative membrane occasioned by the *B. coli* strain. *B. paratyphi* was not recovered from the feces.

Several series of stock guinea pigs, selected at random from the benches, were examined during the endemic period for the fecal carriage of *B. paratyphi*. Of 40 animals cultured the organism was recovered from the feces in only one. This guinea pig was one of a series of 12 removed from a bench shortly after the appearance of a fatal case of paratyphoid in it. The fecal culture was positive immediately after segregation. Cultures made 6 and 9 days later were negative. The animal was killed on the 21st day. *B. paratyphi* was obtained in cultures from the spleen but not in cultures from the feces or in cultures inoculated with a piece of a Peyer's patch.

A survey of the possible agencies transmitting the virus from cage to cage and group to group focused attention upon a few gray mice which gain access from time to time to the building from the fields and woods around it. Data already presented clearly showed that

white mice freely discharge the guinea pig strain for some time after having been fed. Early experience of one of the authors with wild mice indicated no appreciable difference of gray and white mice towards hog cholera bacilli. The agency of wild mice was furthermore suggested by the occasional occurrence of a paratyphoid disease among breeding rabbits kept in another wing of the same building. The cases were rare, not more than several in the fall of 1924, a few in 1925, and one in April, 1926. The lesions were characteristic of the less acute type of paratyphoid infection in rabbits, such as necrotic follicles in appendix, necrotic follicles in Peyer's patches, enlarged, firm, congested spleen, and, very rarely, necrotic foci in liver. The isolated bacilli agreed culturally and serologically with the guinea pig strain. Several wild mice examined recently did not carry *B. paratyphi* however.

DISCUSSION.

Spontaneous epidemics among small animals have not been studied in the past with the care warranted by the importance of the phenomenon. Most investigators have perhaps reached the conclusion that the many unknown factors in spontaneous epidemics stand in the way of any trustworthy conclusions to be derived from such study. Hence the experimentally controlled epidemiological investigations of recent years (Topley (4), Amoss (3), Webster (5)). Nevertheless it is the natural epidemics from which must be drawn the problems to be solved. Without such study the problems do not readily present themselves or they may be overshadowed by the artificial conditions of the experiment. In these pages by the natural epidemic is meant the occurrence of disease without the use of cultures artificially administered. In the epidemic reported above the question as to the decline of the epidemic to the endemic level was uppermost. Among the hypotheses to present themselves the following appear to us as involving the major factors.

(*a*) The sudden, acute, highly fatal onset of the infection may be interpreted as an attack upon a number of animals below the normal resisting power of the species which had accumulated during the 6 years of freedom from disease. This accumulation may be due to fortuitous sexual selection going on in the population or to spon-

taneous variation in natural resistance. We may assume that most of the individuals belonging to this category were wiped out in the early weeks of the disease when the breeders were the chief victims. This theory is supported by the fact that no epidemic rise has occurred since the fall of 1924. The occasional appearance of an animal with marked lesions may be due to individually depressing environmental causes, to which the group is subject, as well as to a reappearing, less resistant stock.

(*b*) Another hypothesis assumes an increase in specific immunity towards *B. paratyphi* following the ingestion of minute doses which caused the epidemic to subside towards the endemic level. This theory is not well supported. There should have been found a less abrupt drop in mortality and many more carriers in the exposed guinea pigs during the endemic period. It is not to be denied, however, that the resistance of certain groups may have been raised by the ingestion of minute doses in the early epidemic phase.

(*c*) A third hypothesis assumes a decline in the virulence of the bacillus in passages through successive guinea pigs. Stated differently, this hypothesis assumes that the virus was introduced from a somewhat higher level of virulence, possibly from some other host species, and after several passages was thereby brought into equilibrium with the new host. This hypothesis was tested in several ways. A culture from one of the earliest acute cases was at hand to be tested comparatively with a culture from the later endemic period. The former had been isolated July 14, 1924; the latter March 19, 1925. The possible unreliability of any comparative tests was kept in mind in view of the additional 8 months of artificial cultivation of the early strain. Subcutaneous and intraperitoneal injection of graded dilutions prepared from 18 hour bouillon cultures of the two strains was made in guinea pigs of approximately 350 gm. weight. With the series which received subcutaneous injection a slight difference in virulence between the two cultures was encountered. The older culture showed a virulence approximately four times that of the endemic culture. A dilution range of the bouillon culture of 1:20 to 1:160 was employed. Within that range extensive ulceration at the site of injection resulted with both cultures. Death occurred in approximately 10 days with the guinea pigs which

had received the older culture in a dilution of 1:80. Of the guinea pigs which had received the endemic culture death occurred only following a dilution of 1:20 after a slightly longer period.

The results obtained from the series which had received intraperitoneal injection of the two cultures were less suggestive of a difference in virulence. The intraperitoneal injection of the older culture in a dilution of 1:500 resulted in death in 48 hours. With the endemic culture death did not occur until 8 days after the injection. Dilutions higher than 1:1,000 produced death in 10 to 14 days or more with advanced focal lesions in the spleen and liver. In several later series which had received graded dilutions intraperitoneally no consistent difference in the lethal dose was observed. The guinea pigs employed in the tests were descendents of the original infected population several generations removed from the active period of the epidemic. Individual differences in natural susceptibility might be sufficient to account for fluctuations in the response to graded dilutions.

A second method of comparing the virulence of the bacillus in the epidemic and the endemic period was to study the lesions of cases occurring in these periods. Tissues from 31 animals were examined in fixed and stained sections. This material consisted of breeders, very young and young adult animals, and of cases from the early epidemic and the late endemic period. The material was from both naturally dead and chloroformed animals. A comparative study of this miscellaneous material did not bring to light any histological characters clearly distinguishing the early and subsequent periods. The various changes in spleen, liver, lymph nodes, and intestines were found at all periods and in various ages and a detailed description of these changes is therefore omitted.

A change in virulence has thus not been demonstrated. It must be granted, however, that the methods employed to show such transformation are relatively crude. The change from an epidemic to an endemic level is obviously due to a number of cooperating and mutually interfering factors and all we can state at present is that it actually occurs in all epidemics sooner or later in a stationary population, in spite of the fact that such groups of bacteria as streptococci, pneumococci, and bipolar organisms (hemorrhagic septicemia)

may be raised in virulence by passages through a series of animals. The increase in virulence of the paratyphoid group by passages is not clearly proved by experiment. Moore (2) failed to increase the virulence of hog cholera bacilli by passages through rabbits. Webster (5) failed to show any rise in virulence. Lockhart (6) concludes that the virulence of a single strain of *B. aertrycke* may be significantly increased as the result of animal passage, but in the text he admits that such increase cannot be brought about with any regularity. A prolonged experience of one of us with the hog cholera bacillus since 1885 leads to the conclusion that passages tend downwards rather than upwards in capacity to kill. If experiments in epidemiology are to elucidate natural phenomena, it is to be borne in mind that passages through a series of susceptible animals by inoculation do not imitate nature, for in the spontaneous disease the infectious agent is transmitted through the digestive tract and of what goes on there nothing is known. That the selective action of the host is the same through enteral and parenteral channels may at least be doubted. Furthermore, virulence is regarded by some as the capacity to multiply and kill, whereas in passages the change may be towards greater tenacity of life in the tissues rather than the capacity to multiply and thus destroy. The infection in the population under consideration has now been followed through three summers, the first being that of the original epidemic period. In 1925, the year following the initial outbreak, the mortality was highest during the summer months, reaching a peak in June, with a rate of 4.1 per cent, followed by a decline in July. For 1926, up to September, the mortality was likewise greatest during the summer with a high rate of 6.4 per cent in August. A rough evaluation of the three summer periods may be made by comparing the combined rates for July and August of each year. It was during that period that the majority of deaths occurred at the time of the original epidemic. Taking into consideration the few deaths that occurred in September, 1924, which were included in the original rate computed for that period, the percentage mortality for July and August, 1924, was 16.7 per cent. The rates for a corresponding period of time in 1925 and 1926 were 3.7 per cent and 6.6 per cent, respectively, definitely lower than the rate of the epidemic period.

The infection has therefore been followed long enough to eliminate the various secondary influences such as changes in the character of the food during the four seasons and changes in humidity and temperature. During the occasional excessive heat periods of the summer months deaths among adult guinea pigs have occurred not referable to anything but the heat.

SUMMARY.

Factors bearing on the maintenance of paratyphoid in an endemic state are discussed. There was no evidence of any increase nor any clearly demonstrable proof of a decline in virulence of the causative organism. This persisted within the breeding stock and it is suggested that the sows constituted the chief focus for dissemination of the organism to their young and from these to the population at large. Evidence is presented that the carriage of *B. paratyphi* in the feces was of relatively short duration. Fecal carriage of *B. paratyphi* was commonly associated with a localization of the organism in the spleen. Since it is obvious that some factor or factors must have changed in the transition from epidemic to endemic phase in the presence of younger generations, the hypothesis is tentatively presented that the transition from epidemic to endemic phase is due to a combination of the weeding out of individuals of low natural resistance with a gradual adjustment of the invading organism to the population on a lowered level of virulence.

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THE BIOLOGICAL CHARACTERS OF A MUCOID VARIANT OF BACILLUS PARATYPHI FROM GUINEA PIGS.

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A mucoid type of *B. paratyphi* was encountered during the course of a spontaneous epidemic of paratyphoid in a previously normal guinea pig population. The active stage of the epidemic occupied a period of 8 weeks in the summer of 1924 and was followed by an endemic stage which has continued with sporadic deaths for 2 years. The normal, smooth type of organism was isolated from the majority of guinea pigs that succumbed to the infection during both stages. Early in the endemic stage, however, two cultures were obtained which differed from the normal in that they were distinctly mucoid and flocculated spontaneously in bouillon. The first culture, No. 1004, was isolated from the spleen of an unweaned, female guinea pig 13 days old. At autopsy the spleen was enlarged, congested, and covered with foci. A mixed growth of the smooth and mucoid types was obtained from the spleen upon plating from the original tissue culture. The second culture, No. 950, was likewise recovered from an unweaned female. The guinea pig died 6 days after birth. At autopsy a slight congestion of the lung was encountered but no typical gross lesions of paratyphoid. A pure growth of the mucoid type was obtained from the spleen.

Mucoid types of the human strains of *B. paratyphi* have been encountered and reported in the literature. Fletcher (1) isolated mucoid varieties of *B. paratyphosus* b from the feces of two chronic carriers and of *B. aertrycke* from the feces during an acute case of meat poisoning. The variants were motile, often small, coccoid bacilli which produced a mucoid substance apparently not as a true capsule and formed large, wet colonies on agar. They were agglutinated in low dilution by an antiserum specific for the normal type. One of the *B. paratyphosus* b variants did, however, absorb the agglutinins from the serum. The other did so imperfectly.

There was no absorption by the *B. aertrycke* variant from an "*aertrycke*" serum. After several daily transplants in peptone water followed by plating the mucoid varieties gave rise to varying numbers of normal colonies. The normal type could likewise be recovered from agar slants of the variant which had been held for several days. The mucoid type was never recovered from the normal, non-mucoid culture.

Thjøtta and Eide (2) isolated a mucoid variant from the urine of a chronic paratyphoid patient. The organism was small, showed few motile forms, and produced a mucoid intercellular substance. It formed large, rounded, wet colonies on agar. Its agglutinability and absorptive capacity were judged to be comparable with those of the normal type, except that the reactions were slower. The mucoid character was regarded as constant although serial plating from single colonies showed at first the production of a small number of normal colonies. The variant was slightly less virulent for mice, upon injection, but the difference was not held to be significant.

Walker (3) found that the prolonged cultivation of *B. paratyphosus* b in bouillon containing specific antiserum gave rise to the production of a mucoid type of the organism. The variant was almost completely non-motile and formed large, umbilicated, moist, waxy colonies on agar. It was agglutinated in low dilution only by *B. paratyphosus* b antiserum. An old agar culture of the variant was transplanted daily in bouillon and reverted to the normal type losing its mucoid nature and becoming motile and agglutinable.

Krumwiede, Cooper, and Provost (4) encountered mucoid variants of *B. paratyphosus* b in the feces of paratyphoid carriers. The mucoid type showed some tendency to produce smooth and rough varieties on cultivation. Absorption tests with the mucoid variant gave anomalous results. It showed little ability to absorb the agglutinins from "rough" and "smooth" antiserums. The low agglutinability and absorptive capacity indicated that the mucoid type differed antigenically from the rough and smooth types of the same strain. It was not believed that the antigenic difference was due solely to the mucoid characteristic.

In Germany considerable attention has been directed towards the appearance of so called *Schleimwall* colonies in cultures as a means of differentiation between *B. paratyphi* b Schottmüller and the meat-poisoning types, *B. enteritidis* Gaertner and Breslau. Freshly isolated cultures of the former when kept at room temperature after 24 hours of incubation at 37°C. were regularly found to form colonies which showed an outer zone or wall radially striated and slimy in nature due to the development of an intercellular material. Such colonies were supposed to be imperfectly developed with the Gaertner strains and absent with the Breslau strains. A critical review of the phenomenon together with a discussion of the rôle of chemical stimuli, such as sodium chloride and increased H ion concentration, in initiating the growth is given by Elkeles (5, 6).

The two mucoid variants under discussion were short, rather plump bacilli. With ordinary methods of staining and in wet preparations

they showed little deviation in size from the smooth type. Sometimes the latter appeared a trifle longer, more distinctly rod-shaped, while the mucoid types more closely approached a coccoid shape. Films made from an 18 hour agar growth fixed with acetic acid and stained with carbolfuchsin showed a faintly stained envelope of indefinite outline surrounding individual cells or pairs of cells. With heavier films the bacteria appeared embedded in a matrix of similarly stained material. With definitely encapsulated bacteria the acetic acid-fuchsin method showed an envelope with distinct boundary. As noted by other workers, the surrounding envelope displayed by the mucoid variants was more in the nature of a viscid intercellular substance than a true capsule. The same method applied to films made from the smooth type showed no enveloping material. Whatever the nature of the envelope it did not appear to affect materially the motility of the organisms. 6 hour bouillon cultures of the mucoid variants showed fine clumps of bacteria together with many free forms. The latter were very actively motile. The clumps also displayed considerable motility. With continued incubation the clumps increased in size and tended to become fixed. Small numbers of free forms were always observed, through 36 hours growth, and these retained their motility.

The mucoid variants gave biochemical reactions identical with those of a smooth type of the epidemic strain. The three cultures fermented dextrose, maltose, levulose, galactose, dulcitol, arabinose, mannitol, and xylose with the production of both acid and gas. Neither acid nor gas was produced from saccharose, lactose, and salicin. Hydrogen sulfide was formed in peptone media but no indole. Gelatin was not liquefied. A strong terminal alkalinity was produced in milk attended by a partial clearing of the medium which presented a slightly yellowish, semitranslucent appearance after 10 days.

The mucoid types differed markedly from the smooth type in the character of growth in bouillon and on agar. The smooth type gave rise to a heavy, even turbidity in bouillon with little tendency towards settling except upon prolonged cultivation. Sometimes a slight scum formed on the surface of the medium but a definite, complete film was never observed. With the mucoid types a spontaneous flocculation followed by sedimentation occurred in bouillon. A 48

hour culture showed a moderate to heavy sediment, a slightly turbid supernatant with floccules in suspension, and generally a distinct surface film.

On agar the smooth type formed transparent, flat, moist but non-mucoid colonies which had a bluish cast and regular border. The colonies reached a size of 4–5 mm. after several days on a thinly seeded plate and tended to become slightly convex showing a deeper colored central zone which gave them a ringed appearance. The mucoid types both formed slightly larger colonies which reached a size of 5–6 mm. after 4 days at room temperature. Sometimes the growth was stringy but more often waxy in nature forming heaped up masses when touched with a needle. The colonies usually became differentiated into a central zone which was either wrinkled with radial striations or granular, and a smooth, outer rim of varying width. At times part or all of the colonies showed only a raised, smooth, central zone. Such colonies when picked into bouillon and plated gave rise to the wrinkled and granular type as frequently as they did to the undifferentiated type of colony.

On agar slants the mucoid types produced a heavy growth which was stringy in nature. On older slants the growth became wrinkled and less definitely viscid. Saline suspensions were formed with difficulty and with the usual salt concentration spontaneous flocculation always resulted. In distilled water and in solutions of reduced salt concentration, 0.4 per cent and less, the suspensions were more stable and showed only a scant sediment upon standing.

The experience of other workers with mucoid variants of the human strains of *B. paratyphi* indicated a tendency towards reversion to the normal type upon culturing. Likewise there appeared to be a tendency for the normal strains, in old culture or under special conditions of culture, to split off mucoid types in varying numbers. Inasmuch as one of the present guinea pig variants was isolated together with the normal type of *B. paratyphi*, it was regarded of interest to determine whether a similar reversion and splitting occurred with the animal strain. The presence of the variant in the guinea pig host might be explained by such a splitting from the normal type of organism.

The two mucoid types were plated on agar from 18 hour bouillon

cultures, a single colony of each type picked into bouillon and again plated after 18 hours. The procedure was repeated through 12 generations, 6 in bouillon and 6 on agar. The bouillon tubes always showed the characteristic growth with sediment, spontaneous flocculation, and surface film. On agar there were produced only waxy or viscid colonies with or without differentiated zones. Daily bouillon cultures of the two types were made through 30 generations and plated at intervals. The results were the same. The characteristic growth was maintained in bouillon and on agar.

The intraperitoneal injection of the mucoid types in low dilution into guinea pigs resulted in death and the recovery of the organism from the visceral organs and the peritoneal exudate. The growth was typically mucoid on agar slants and transplants in broth yielded the usual flocculating growth which on plating produced only waxy or viscid colonies. Similar results were obtained with cultures isolated from rabbits and from mice which had received intraperitoneally fatal doses of the organism.

There was no indication that repeated culturing of the mucoid variants in ordinary media caused a reversion to the normal type. Nor did there appear to be any reversion attendant upon the growth of the variants under parasitic conditions in experimental animals.

Six normal strains of *B. paratyphi*, isolated from guinea pigs which had succumbed to paratyphoid, were similarly carried through 8 successive generations on media, 4 in broth and 4 on agar. The bouillon tubes invariably showed an even turbidity with no flocculation and no surface film. On agar the colonies were all of the same smooth, non-mucoid type. A single strain was carried through 30 generations in bouillon with intermittent plating for colony characteristics. A change occurred in the nature of the growth in bouillon during the series of transfers. The culture developed a surface film and showed a slight tendency towards flocculation with increased sediment. On agar, however, there was no change noted in the nature of the colonies. They were always of the smooth, non-mucoid type.

The same culture was carried through 3 generations in bouillon containing 10 per cent specific antiserum and in 5 per cent peptone water. The transfers were made every 4th day. Plates were poured

from the two kinds of media on the 4th day after the third transfer. In both instances the plates showed two types of colonies in approximately equal numbers, the typical smooth colony and a more opaque type with an irregular, waxy border. None of the colonies were mucoid. The first type produced the usual even turbidity in bouillon. The second type showed spontaneous flocculation in bouillon with sediment but no surface film.

On several occasions cultures were obtained from the spleens of guinea pigs which had received intraperitoneally non-fatal doses of the smooth type of *B. paratyphi*. The spleen, liver, and lymphoid tissue of the intestinal tract gave evidence grossly of some structural change. The blood serum showed a moderate titer of specific agglutinins. The cultures on plating yielded only the smooth type of colony.

There was no indication that the normal, smooth type of *B. paratyphi*, of the present epidemic strain, yielded mucoid variants either with prolonged cultivation in ordinary media or in special media designed to stimulate variation. Growth in the latter media did, however, give rise to a rough type of variant. Moreover the smooth type failed to show any tendency towards variation in the guinea pig under conditions favorable for variation, that is in animals which had acquired some degree of resistance.

The relationship of the two mucoid types to the smooth or normal type of *B. paratyphi* was studied with the aid of the agglutination reaction. Antiserums were prepared by intraperitoneal injection of the three cultures into rabbits. Serums of high titer were obtained. The tests were made in approximately 0.4 per cent salt solution in order to reduce spontaneous flocculation. Readings were made only after 3 hours of incubation at 37°C. With continued incubation a scant flocculation occurred in the controls of the mucoid types. Direct agglutination tests showed no difference between the two types. The limit of agglutination was the same for each culture with the three serums. No difference was noted in the character of flocculation nor in the velocity of the reactions.

Reciprocal absorption tests were employed with the three cultures and their respective serums. The technic of Krumwiede (4) was followed except that absorption was carried out in 0.4 per cent salt

TABLE I.

*Agglutinin Absorption Tests with Normal and Mucoid Type Antiserums.**

Serum	Culture	Direct agglutination	Absorbing culture	Agglutination after absorption	Culture tested after absorption
Normal 922	Normal 922	1:51,200	Normal 922	1:200	Normal 922
				1:200	Mucoid 1004
				1:200	" 950
	Mucoid 1004	1:51,200	Mucoid 1004	1:400	Normal 922
				1:400	Mucoid 1004
				1:400	" 950
	" 950	1:51,200	" 950	1:400	Normal 922
				1:400	Mucoid 1004
				1:400	" 950
Mucoid 1004	Mucoid 1004	1:51,200	Mucoid 1004	1:800	Mucoid 1004
				1:800	" 950
				1:800	Normal 922
	" 950	1:51,200	" 950	1:800	Mucoid 1004
				1:800	" 950
				1:400	Normal 922
	Normal 922	1:51,200	Normal 922	1:400	Mucoid 1004
				1:400	" 950
				1:400	Normal 922
Mucoid 950	Mucoid 950	1:51,200	Mucoid 950	1:400	Mucoid 950
				1:400	" 1004
				1:400	Normal 922
	" 1004	1:51,200	" 1004	1:400	Mucoid 950
				1:400	" 1004
				1:400	Normal 922
	Normal 922	1:51,200	Normal 922	1:400	Mucoid 950
				1:200	" 1004
				1:200	Normal 922

* The absorbing dose was 1:5 and the serum dilution 1:10 throughout.

solution and final readings made after 3 hours of incubation at 37°C. The results of the absorption tests are given in Table I. No outstanding difference in absorptive capacity was displayed by the normal

and mucoid types of *B. paratyphi*. The variants were, however, a little less efficient in the removal of agglutinins from the three serums. The variation in absorptive capacity did not appear sufficiently great to indicate an actual antigenic difference between the two types.

TABLE II.

Preliminary Virulence Test with the Normal and Mucoid Types of B. paratyphi.

Culture	Dilution	Result	Spleen culture
Normal 922	1:2,000	Died 4 days	+
	1:20,000	" 10 "	+
	1:200,000	" 16 "	+
Mucoid 1004	1:2,000	" 10 "	+
	1:20,000	Killed 21 "	−
	1:200,000	" 21 "	+
" 950	1:2,000	Died 10 "	+
	1:20,000	Killed 21 "	−
	1:200,000	" 21 "	+

TABLE III.

Final Virulence Test with the Normal and Mucoid Types of B. paratyphi.

Culture	Dilution	Result	Spleen culture
Normal 922	1:200,000	Died 5 days	+
	1:200,000	" 6 "	+
	1:200,000	" 14 "	+
	1:200,000	Killed 26 "	+
Mucoid 1004	1:200,000	" 26 "	+
	1:200,000	" 26 "	+
	1:200,000	" 26 "	+
	1:200,000	" 26 "	−
" 950	1:200,000	Died 15 "	+
	1:200,000	Killed 26 "	+
	1:200,000	" 26 "	−
	1:200,000	" 26 "	+

A comparative study of the virulence of the normal and mucoid types was made on guinea pigs. As a preliminary graded dilutions

prepared from 18 hour bouillon cultures were injected intraperitoneally into guinea pigs of approximately 350 gm. weight. Little difference was noted in the number of bacteria per cc. with the three cultures. Plate counts gave roughly 600,000,000 bacteria per cc. for each. Dilutions of 1:2,000, 1:20,000, and 1:200,000 in 1 cc. of diluent were injected. One guinea pig only was injected with each dilution. The results of the test are given in Table II.

The preliminary test indicated a considerable difference in virulence between the two types. As a final check four guinea pigs were injected intraperitoneally with 1 cc. amounts of each culture in dilutions of 1:200,000, the highest lethal dose of the normal type. The results of the test are given in Table III.

The final test did not bear out the sharp difference in virulence indicated by the preliminary series. The normal type failed to kill 100 per cent of the test animals in the dilution employed. In addition one of the mucoid types displayed lethal action in that dilution. It seems probable that a difference in virulence does exist in favor of the normal type as the higher, but definite conclusions would be warranted only with a much larger series of animals.

The susceptibility of the mucoid variants to lysis by a filtrate active for the smooth type of *B. paratyphi* was determined. The filtrate employed was obtained from the feces of a mouse infected with paratyphoid through contact with a second mouse which had received *per os* administration of *B. paratyphi*. The method of serial dilution in bouillon was used in determining the activity of the filtrate. Dilutions ranging from 10^{-1} to 10^{-12} were made in 5 cc. amounts of bouillon and 0.05 cc. of an 18 hour bouillon culture added to each tube of the series. Readings were made at the end of 3, 6, and 48 hours incubation at 37°C. The end-point was determined by filtering the three tubes of highest dilution and retesting in series. The filtrate was active for its homologous culture, *i.e.*, the smooth type of *B. paratyphi*, through a dilution of 10^{-10} or 10^{-11}. Complete lysis of the culture never occurred. After 3 hours incubation there was no visible turbidity in dilutions of 10^{-1}, 10^{-2}, and 10^{-3}; from 10^{-4} through 10^{-6} there was a graded turbidity, and from 10^{-7} through 10^{-11} the turbidity was in the same as that of the control. After 6 hours incubation there was a faint turbidity in the first three dilutions which increased

in intensity upon further incubation. Resistant and susceptible bacteria could be isolated from any of the tubes in which the filtrate was active. The filtrate was inactive for the mucoid types. There was no inhibition of growth in any dilution. After 3 hours incubation all the tubes showed a distinct, finely floccular growth identical with that of the control. Further incubation resulted only in increased growth normal in appearance for the mucoid type. Plates poured and streaked at varying intervals of growth showed only normal mucoid colonies.

DISCUSSION.

The guinea pig strain of *B. paratyphi* associated with the epidemic and regarded as the normal type appears to differ from the normal strains of human origin in that it displays a less marked tendency towards variation. Other workers have shown that the human strains commonly give rise to variants of the mucoid and rough types. Variation may occur under normal conditions of growth or it may be stimulated by prolonged cultivation and by rapid serial transfer. Alteration in the chemical nature of the medium by the addition of specific antiserum and by increased sodium chloride, peptone, or H ion concentration may also afford a stimulus to variation. The action of a specific lytic principle may likewise result in variation. The normal guinea pig strain has shown no tendency towards a mucoid variation under normal conditions of growth nor under the stimulus of specific antiserum, increased peptone concentration, or the action of a lytic principle. The latter stimuli do, however, initiate a variation resulting in the appearance of the rough type of variant which flocculates spontaneously in bouillon and is resistant to lysis.

Mucoid variants have not been recovered experimentally from the normal guinea pig strain either under parasitic or saprophytic conditions of growth. They have been recovered, however, from the guinea pig in two cases of spontaneous infection. The mucoid types have been compared with the normal strain as to biological characters. In general they display some difference in character from the mucoid types isolated by other workers from the human strains of *B. paratyphi*. The variants of the latter strains were usually found to be less agglutinable than the normal type and to show a tendency

towards reversion upon cultivation. The variants under discussion display little deviation from the normal type in agglutinability or in antigenic properties. The mucoid character of growth appears constant and no tendency towards reversion is displayed.

Experimental study has failed to demonstrate a stimulus which might account for the appearance of the mucoid types in the guinea pig host. If variation within the guinea pig organism resulting in a splitting from the normal type under the stimulus of resistance were accountable, it would seem that the mucoid types should be more frequently encountered. It is believed, rather, that the two types had a more remote, common ancestry and that the mucoid type as a variant of permanent characters was transmitted to the guinea pig population together with the normal type. Such an explanation likewise fails to account for the infrequency of its occurrence. Differences in virulence might be regarded as an associated factor.

SUMMARY.

The biological characters of two mucoid variants of *B. paratyphi* isolated from guinea pigs have been studied and compared with those of the normal type. The possible origin of the mucoid type is discussed.

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VIBRIOS FROM CALVES AND THEIR SEROLOGICAL RELATION TO VIBRIO FETUS.

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Nothing is known of the life cycle of *Vibrio fetus* beyond its presence in the bovine placenta and the fetus following abortion. In the digestive tract of the fetus the vibrio is usually found in pure culture. If the fetus is not invaded, however, the difficulties encountered in the placenta and uterine discharges, owing to contamination with miscellaneous saprophytic bacteria, have made isolation thus far impossible. This is due to its early very feeble multiplication, restricted to sealed agar tubes containing blood or fresh tissues or to tubes in an atmosphere containing a small per cent of CO_2, and its rapid destruction following injection into laboratory animals. The occasional encounter of vibrios in the intestinal tract of young calves calls attention to a possible locus of *Vibrio fetus*. These vibrios might be survivors of a fetal infection with *Vibrio fetus*, or they might represent a different group possibly associated with intestinal inflammation in calves after the 1st week.

The first strain (No. 174) was obtained in pure culture from the spleen of a calf killed when 10 days old. This animal began to scour when 5 days old and when killed was very weak. At autopsy, the middle portion of the small intestine was deeply congested. Fresh villi under the microscope showed the entire capillary network injected and certain groups of epithelial cells undergoing fatty changes. The large intestine contained normal fecal matter but the mucosa was overlaid with stringy elastic masses of mucus. *Bacillus fluorescens* was isolated from the intestines on agar plates. No special search was made for vibrios. In sections of small and large intestines, vibrios were not found. Agglutination relations of this strain have been briefly described.[1] Sera prepared with three different living cultures of *Vibrio fetus* failed to act upon this strain, although

[1] Smith, T., and Taylor, M. S., *J. Exp. Med.*, 1919, xxx, 299.

clumping *Vibrio fetus* almost completely at 1:640 dilution. A specific serum prepared with the calf strain clumped it at 1:2,560 completely but failed to clump fifteen strains of *Vibrio fetus* at 1:20.

A second vibrio (No. 321) was isolated from a calf dead when 58 days old and noticed sick for about a week. The autopsy showed small ulcerations of rumen and the traces of early hemorrhages of the fourth stomach mucosa.[2] There was considerable mucus in the small intestine and the large intestine was congested. There was pneumonia of the left lung indicated by numerous, small, partly coalescing foci of a flesh red color. In sections these foci consisted of injected capillaries and alveoli filled with polymorphs. There was a large amount of coagulable protein in the urine.

The vibrio appeared in cultures of the liver, and in a spleen culture of a guinea pig killed 7 days after inoculation with contents of duodenum. The serological relation of this strain to *Vibrio fetus* has been briefly given.[1] It agrees so far as direct agglutination goes with *Vibrio fetus*. Morphological and cultural distinctions could not be demonstrated.

The two strains described were isolated in 1917 and 1918. In 1925 an obscure disease of calves was brought to our attention. No study of the disease was made on the spot and the one suggestive fact reported was that the calves had been bedded in buckwheat hulls. Two calves, one living, one dead, were brought for examination.

No. 1206.—Ayrshire female, about 3 weeks old and weighing 64½ pounds. Dried feces on buttocks and tail. Kept under observation 15 days when the calf died. During this period it was alternately constipated and passing watery feces. The temperature fluctuated around 39°C. There were signs of pneumonia. Milk was taken in small quantities.

Autopsy.—The rumen distended during life was about twice normal size and filled with cut straw, buckwheat shells, and hair. There was some congestion of large intestine. The cephalic lobe of the right lung was consolidated, larger than normal, and permeated with numerous grayish foci, 2 to 10 mm. in diameter. The pneumonia was associated with a bipolar organism (*B. bovisepticus*). Cultures with bits of liver, spleen, and kidneys remained sterile with the exception of one liver tube which contained a vibrio.

No. 1208.—Guernsey female, brought to the Department about 12 hours after death. Age, 23 days. Weight after death, 59 pounds. The report was that the calf had developed scours. There was pneumonia of the cephalic and ventral lobes of the right lung similar in character to that of No. 1206, and the same bipolar type of organism was isolated. The intestines were more or less congested. Cultures of spleen, liver, and kidneys were negative like those of No. 1206 with

[2] Florence, L., *Am. J. Dis. Child.*, 1922, xxiii, 132.

the same exception that in one containing liver tissue a vibrio appeared in culture obviously like that from No. 1206. Films and sections of the intestines did not show vibrios.

At this time a small number of strains of *Vibrio fetus*, some isolated recently, others several years before, were on hand. Strains 174 and 321 had died out. Cultural differences between Strains 1206 and 1208 and the fetal strains were limited to a freer, more rapid multiplication on agar slants of the former. Agglutination tests were therefore undertaken. The vibrio cultures studied included five fetal strains and two calf strains. The cultures were grown on agar slants sealed with sealing wax. The growth was used after 3 to 4 days incubation at 37°C. The immune sera were prepared by in-

TABLE I.

Cross-Agglutination between Living Fetal and Calf Strains in Serum of Rabbits Immunized with Living Cultures and Cultures Heated at 100°C. for 2 Hours.

Rabbit serum prepared with	Agglutination titer						
	Fetal strains					Calf strains	
	741	996	997	1149	1217	1206	1208
997 (living)	1:2,560	1:5,120	1:10,240	1:5,120	1:5,120	1:1,280	1:2,560
997 (heated)	1:20	1:5,120	1:2,560	1:2,560	1:2,560	1:20	1:20
1208 (living)	1:2,560	1:2,560	1:1,280	1:2,560	1:1,280	1:2,560	1:10,240
1208 (heated)	1:20	1:20	1:20	1:40	1:20	1:2,560	1:2,560

jecting rabbits intraperitoneally. The injections were made with living cultures and with cultures heated 2 hours at 100°C. Living cultures, standardized by the Gates instrument to disappear at 2.4 on the scale, were used for the agglutinating and absorbing suspensions. The absorbing dose used was 6 times the concentration as represented by 2.4 on the Gates scale, *i.e.* 6 times the agglutinating dose. Each serum was absorbed by the same dose of each culture so that the results are comparable.

The experiments include cross-agglutinations with sera prepared by injecting living and heated cultures and reciprocal absorption tests carried out between calf and fetal strains, between fetal strains, and between calf strains. In Table I, the various relationships be-

tween fetal and calf strains tested with the sera of rabbits treated with living and heated cultures are briefly summarized.

With one exception, the fetal strains present no differences among themselves when tested with "living"[3] or "heated" sera of fetal strains. This exception is No. 741. In the "heated" sera of 997 only a little agglutinin for 741 was present. When the fetal strains were acted upon by "living" and "heated" sera of calf strain 1208, only the "living" serum was found to contain agglutinins for them. The calf strain agglutinins were only slightly lower in the "heated" calf strain serum than in the "living" serum. When the calf strains were treated with "living" and "heated" sera of the fetal strain 997, it was found that the agglutinins common between calf and fetal strains were present only in traces in the "heated" serum. There was therefore no cross-agglutination between calf and fetal strains when tested in "heated" sera. This test defines two serological groups based on the habitat of the strains. It indicates the existence of a distinct heat-stable calf-group antigenic factor and a distinct heat-stable fetal-group antigenic factor.

Absorption tests were next tried with results as given in Table II. In making and interpreting the absorption results the statements of Krumwiede, Cooper, and Provost[4] on agglutinin absorption were taken into consideration. These are in substance that direct agglutination may not be a reliable index of the serological relationship of a bacterium. Absorption of common agglutinins cannot be a criterion for likeness but such an absorption may suggest groupings. To determine agglutinative identity it is necessary to demonstrate specific agglutinins. The reciprocal absorption test is therefore the ultimate method available for determining agglutinogenic likeness or unlikeness. A complete reciprocal reaction indicates identity and the absence of such reciprocal reaction indicates dissimilarity. Between these extremes there may be found all degrees of partial reciprocal reactions.

Table II illustrates the different types of reciprocal absorption reactions which occurred between calf and fetal strains, between

[3] These abbreviated terms signify "sera from rabbits injected with living vibrios or vibrios exposed to 100°C. for 2 hours."

[4] Krumwiede, C., Cooper, G., and Provost, D. J., *J. Immunol.*, 1925, x, 55.

individual fetal strains, and between individual calf strains. The readings given are those made after three successive absorptions. The results throughout each series were very similar, so that the examples tabulated cover all the different degrees of reciprocal absorption observed.

A certain serological difference between calf and fetal strains was

TABLE II.

Examples of Reciprocal Absorption Tests.

Serum	Absorbed by	Agglutinated by		Titer before absorption	
1. Between calf and fetal strains					
		1208	997	1208	997
1208 (calf)	997	1:5,120	1:20	1:10,240	1:1,280
997 (fetal)	1208	1:160	1:10,240	1:2,560	1:10,240
2. Between individual fetal strains (1149 and 997)					
		1149	997	1149	997
1149	997	1:40	1:20	1:1,280	1:640
997	1149	1:320	1:320	1:5,120	1:10,240
				(996 and 997)	
		996	997	996	997
996	997	1:640	1:40	1:5,120	1:5,120
997	996	1:320	1:320	1:5,120	1:10,240
3. Between individual calf strains					
		1208	1206	1208	1206
1208	1206	1:640	1:40	1:10,240	1:2,560
1206	1208	1:80	1:640	1:10,240	1:5,120

demonstrated by reciprocal absorption tests using sera prepared with living organisms. The results showed an absence of reciprocal absorptions and suggest distinct calf and fetal group antigenic factors. Thus by two methods serological differences according to source have been demonstrated.

Reciprocal tests between fetal strains acted upon by sera prepared with living cultures show partial absorptions and almost complete

reactions, indicating an essential identity of fetal strains but possibly some individual strain factor as well. Three strains are practically identical according to the reciprocal tests; namely 997, 1217, and 1149. Strain 996, on the other hand, shows more strain individuality. The 996 agglutinins after three successive absorptions by the other strains are not removed beyond a certain point, while the agglutinins for these other strains continue to be reduced. This indicates some individual strain antigenic factor in 996 which produced these specific agglutinins. The other fetal culture, 741, is an exceptional strain. In absorption experiments it fits into the fetal group, but it differs from the other fetal strains as already mentioned by failing to agglutinate in the 997 serum prepared with the heated culture (Table I).

TABLE III.

Absorption of Agglutinins between the Calf Strains in Sera Prepared with Living and Heated Antigens.

Serum prepared with 1208	Absorbed by 1206	Agglutination titer after absorption		Agglutination titer before absorption	
		1206	1208	1206	1208
Living culture	3 successive times	1:40	1:640	1:2,560	1:10,240
Culture heated at 100°C., 2 hrs.	3 " "	1:20	1:40	1:2,560	1:2,560

The reciprocal tests between the calf strains with sera prepared from living cultures show a definite relation between these strains and also a strain individuality. Each strain absorbs agglutinins for the other up to a certain point, but after three successive absorptions specific agglutinins still remain for the antiserum-producing strain while agglutinins for the absorbing strain are almost wholly removed.

Comparative absorption tests between the two calf strains have been made also with sera prepared with living cultures and cultures heated at 100°C. The results are summarized in Table III. These indicate that the individual strain factor is heat-labile. With the living culture antiserum 1208 repeated absorptions by 1206 left a residue of agglutinins for 1208, while agglutinins for 1206 were wholly removed; but with heated culture antiserum 1208 repeated absorptions by 1206 removed agglutinins for 1208 and 1206 to about the same degree. Thus in the heated culture antiserum there is no spe-

cific strain agglutinin corresponding to that in the living culture antiserum; the individual strain factor is therefore heat-labile.

The relation of the antigenic factors to flagellar and somatic agglutinins has not been demonstrated since no non-motile vibrio form has been isolated. However, since the vibrio strains all show some motility, flagellar and somatic antigens are assumed to be present in all cultures and they may or may not be the basis of the serological difference between calf and fetal strains. Previous work has shown that flagellar antigen in the group of paratyphoid bacilli is heat-labile and somatic antigen heat-stable. The common and the individual strain heat-labile, antigenic factors in the vibrios probably represent the flagellar portion of the antigen and the specific calf and fetal group factors the somatic portion.

In brief these results indicate that at least four types of antigenic factors exist among the vibrio strains studied and any one strain may contain three of these different factors. They may be designated as (1) common vibrio factor, (2) calf group factor, (3) fetal group factor, (4) individual strain factor. The cross-agglutination tests with sera prepared with living cultures give an indication of the common vibrio factor. The cross-agglutination tests with sera prepared from heated cultures and reciprocal absorption tests between calf and fetal strains demonstrate the distinct calf and fetal group factors. The reciprocal absorption tests between fetal strains and between calf strains suggest some individual strain factors. Comparison of results with sera prepared with living and heated cultures indicate that the common vibrio factors and the individual strain factors are heat-labile while the specific calf group and specific fetal group factors are heat-stable. This partial analysis of the antigenic factors demonstrates that the vibrios are of a complex antigenic nature. The serological tests further indicate a close relationship between but not an identity of the calf and the fetal strains.

SUMMARY.

The calf vibrios thus far studied include one strain serologically distinct from the fetal strains. The others are closely related to the fetal strains though not identical with them. The pathogenic characters of the calf vibrios, either as possible descendents of *Vibrio fetus*, or as independent factors in the production of enteritis have not been demonstrated.